ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

FISCAL YEAR 1971

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
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U.S. NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

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NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

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ANNUAL PROJECT REPORTS

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ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR

National Institute of Arthritis and Metabolic Diseases

Fiscal Year 1971

by

Dr. J. E. Rall

This summary of reports from the various Laboratories and Branches of the National Institute of Arthritis and Metabolic Diseases at first glance can easily seem nothing more than a pot pourri of a hundred different and unrelated investigations. The discerning reader, however, may detect several overall themes running through these reports. The first, and one of the more important, is the fundamental unity of biology so that biological principles more often than not can be widely generalized. Enzymes from human liver and from bacteria usually have very similar structure. Major metabolic pathways in all animals are very similar. The genetic code, whenever tested, is identical in all forms of life. The sum of the intricate chemical reactions resulting in life appears to require certain reactions which all forms of life must use.

Another underlying theme is the conviction that biological systems can be explained in physical, chemical and mathematical terms. This leads in many instances to detailed and frequently complicated investigations of, for example, electric dichroism of charged polymers, x-ray crystallography, or reaction rate kinetics which may seem unrelated to clinical problems. A further look suggests that these are attempts to get at deeper explanations and insights into chemical or biological processes so that more rational diagnosis of more effective therapy can be devised. The investigation of chromatin (which contains the basic genetic material) at the moment is concerned with the fraction of DNA which is covered, partially covered or uncovered, by histones or other nucleoproteins and might seem unrelated to clinical problems. But both developmental anomalies and carcinogenesis can be seen as problems in DNA transcription, and transcription is presumed to be inhibited in a large portion of DNA of animals by repressor nucleoprotein bound to DNA.

At another level is the first x-ray diffraction analysis and the first convincing electron microscopic analysis of gamma globulin, the major circulating immune protein. Similarly, a detailed analysis of the protein characteristically deposited in the disease amyloidosis has now shown that it represents part of one of the immunoglobulin molecules. In an important group of congenital metabolic defects, the mucopolysaccharidoses, it is now reasonably clear that soluble factors found in tissue culture media, in the blood, and in higher concentration in the urine, have a curative action on fibroblasts from these patients grown in vitro.

At the clinical level, a new therapy for systemic lupus erythematosus derived from extensive studies in animals with a disease very similar to the disease found in man, has been devised. A new therapy for hyperthyroidism, using the common inorganic compound lithium carbonate, has been shown to be effective.

Philosophically, these reports may be considered the expression of an essentially pragmatic approach to biomedical problems. Some diseases and some biological problems are reasonably well understood; more are shrouded in varying degrees of mystery. To work effectively, to produce results of value, to attempt to reveal major new insights, we must operate at all levels of approach. Fundamental physical and chemical approaches are more appropriate in certain problems; in others, quite practical clinical experiments are necessary. There is no general theory of biological function and malfunction which we call disease, so that a rationale of attack, logical on a priori grounds, can be constructed. Furthermore, the enormous complexity of biological function is such that no one can be expected to encompass all this knowledge and effectively direct detailed individual investigation. On the other hand, experts in the various fields understand enough of the diseases we are attempting to cure to apply their very special knowledge to illuminating the dark masses of ignorance presently inhibiting practical progress in therapy. This report is the story of these investigations.

ANNUAL REPORT SUMMARY

LABORATORY OF NUTRITION AND ENDOCRINOLOGY, 1971

OFFICE OF THE CHIEF

Large-Scale Separations Unit

The number and diversity of requests by NIH investigators continues to increase, occasionally resulting in 3-4 week delays in processing requests. Construction of new facilities has begun and occupancy of the new quarters is scheduled for the last half of 1972.

During the past year, 298 requests by NIH investigators were processed. 165 kilogram quantities (52,000 liters of cultures) were produced from 17 microorganisms, including protozoa, yeast and bacteria and consisted of regular and mutant strains of *Acanthamoeba castellanii, Aerobacter cloacae, Bacillus amyloliquefaciens, Bacillus subtilis, Clostridium thermosaccharolyticum, Comamonas sp., Escherichia coli, Hemophilus influenzae, Methanococcus vannielii, Paramecium aurelia, Pseudomonas acidivorans, Pseudomonas purtida, Pseudomonas sp., Pseudomonad GS, Saccharomyces cerevisiae, Salmonella typhimurium and Alpha Streptococcus.* Some of the organisms were used as hosts for bacteriophage production such as Q beta, Lambda, MS-2, T4, and T7 and others, such as *B.amyloliquefaciens* and mutant strains of *E. coli* were used to produce extracellular enzymes.

Optimum growth conditions for maximum polysaccharide *H. influenzae* were developed during 11 fermentations in 10- to 300-liter runs for the NICHD. Cross-reacting polysaccharide was prepared from two 50-liter cultures of *E. coli* and a simplified method for the large-scale production of a heat sensitive component of the medium was investigated.

Hepatoma tissue cells were successfully grown once in 10-liter and twice in 50-liter batches. Attempts were made to recover aseptically and to reuse the expensive spent medium obtained during the separation and recovery of cells in a continuous flow process.

Large scale processing activities consisted of isolation of cell nuclei, Hurler Factor, of histamine methylating enzyme, RNase inhibitor, aminotransferase, EXO-3 nuclease, polynucleotide phosphorylase, and yeast folates. The Gaulin homogenizer was used to rupture suspensions of yeast or bacterial cells. The large capacity and turbine Sharples centrifuges were used to separate cells from cultures. Eight gallons of hydroxylapatite were made and 56 liters of Leventhal base were prepared from horse blood. Animal diet and various quantities of seeds and resins were ground in the laboratory mills. Ether and alcohol extracts of plant material were concentrated in vacuo in the explosion-proof area and radioactive sweat water was concentrated below room temperatures in the high capacity vacuum system. (D. L. Rogerson, Jr.)

Germfree Research Unit

Studies of the effect of cycasin in germ-free rats have shown that other

young (up to 25 days), in contrast to adult animals, have the ability to convert cycasin to its toxic aglycone form.
(G. Laqueur, (LEP-NIAMD); M. Spatz (LEP-NIAMD) and E. G. McDaniel).

Germfree rats, in contrast to conventional and ex-germfree rats, are able to survive up to a year without demonstrable amounts of vitamin A in their diet. Dry preheating of dietary components (cornstarch and casein) suggests that the difference noted between germfree and conventional rats results from differences in their efficiency in utilizing trace amounts of vitamin A, and not to the lack of a requirement of this vitamin for survival. The early death of vitamin A deficient conventional animals is the result of infection by specific organisms rather than the lack of the vitamin itself. On the other hand, germfreeness in chickens, does not afford protection against vitamin A deficiency either with respect to growth, survival, onset or severity of the symptoms. (J. Bieri, W. Rogers (NIDR) and E. G. McDaniel).

Study of the effect of the intestinal bacteria on the deficiency symptoms of the trace element zinc, has shown that the early death observed in conventional zinc-deficient rats may be due, in part, to infection. Increased survival in germfree rats could be the result of more efficient utilization of traces of dietary zinc and by other dietary minerals. Poor growth observed in rats fed autoclaved egg white diets could be partially overcome by supplementation with threonine. It was found that irradiated egg white diets provide growth comparable to that of raw egg white, which is very low in zinc. Work has been hampered somewhat by the occurrence of kidney stones in germfree rats on low zinc diets. (J. Smith, VA Hospital, and E. G. McDaniel).

SECTION ON DEVELOPMENTAL BIOCHEMISTRY.

Properties and Biological Activity of Oligonucleotides.

Ribonucleotide trimers terminating in 3'-phosphates have been prepared by chromatographic isolation from *Ustilago* RNase digests of yeast. This enzyme produces preferential cleavage -Ap-Pyp linkages. The following additional trinucleotides have been obtained in good purity ACU, AUC, (UC)A, UUA, (UA)U, (CU)G, (UG)U, UGG, and CGG, which, with trinucleotides already in hand, constitute complimentary triplet pairs. Attempts to prepare crystals suitable for X-ray crystallography have, so far, been unsuccessful.
(P. Woodford, G. W. Rushizky, and H. A. Sober).

Dinucleotides (ApA and CpA) coupled to bovine serum albumin via the periodate oxidation procedure produced antisera in rabbits which precipitated specifically with the appropriate dinucleotide attached to a heterologous carrier. Precipitation could be inhibited by free dinucleotides. Both antisera were able to inactivate adenosine-T4 conjugate, indicating antibody specificity to single nucleosides. However, high concentrations of adenosine alone did not inhibit the precipitin reaction. Trinucleotides are being tested as immunogens. (M. Sela, Weizmann Institute, Israel and H. A. Sober).

Structure and Function of Nucleic Acids.

Various systems were examined with a view toward developing an improved

fractionation procedure for tRNAs as well as other oligonucleotides of similar molecular weight. The best separations of charged tRNAs were obtained with a modification of the acid pH gel catalyst of Raymond with 8-12% polyacrylamide gels in 3-6 M urea, at pH 3.2 - 4.2. Separations of uncharged tRNAs were also obtained in the presence of 0.01 M Mg^{2+}, or when the tRNAs were complexed with Hg^{2+}.

Column chromatography of oligonucleotides on DEAE-Sephadex in 7 M urea affords separations according to chainlength only. However, the use of 50% MeOH at pH 5.2 instead of urea permits fractionation of oligonucleotides not only by chainlength but also on the basis of Gp content. This is useful for the fractionation of RNA digests containing compounds of equal chainlength, but differing Gp content. Pure 3'-terminal phosphate forms of ACC, UCC, UUC, AUC,(CGC),(UGA), (UGC), CGG, AGG, and UGG were thus isolated from micrococcal nuclease digests of RNA.

DNA oligonucleotides can also be fractionated on DEAE-Sephadex in 50% MeOH from nonspecific enzymatic digests of DNA. Preparative amounts have been prepared of DNA trimers (ACC), (AAC), TCC, AAA, (ACT), TTC, AAG, (AGC), (CGC), (TGA), (TGC), TTG, CGG, AGG, and TGG. (G. W. Rushizky and J. H. Mozejko).

Structure and Function of Barnase, an Extracellular Ribonuclease from *Bacillus amyloliquefaciens* and its Intracellular Inhibitor, Barstar.

The amino acid sequence of barnase is:

H-Ala-Gln-Val-Ile-Asn-Thr-Phe-Asp-Gly-Val-Ala-Asp-Tyr-Leu-Gln-Thr-Tyr-His-Lys-Leu-Pro-Asn-Asp-Tyr-Ile-Thr-Lys-Ser-Glu-Ala-Gln-Ala-Leu-Gly-Trp-Val-Ala-Ser-Lys-Gly-Asn-Leu-Ala-Asp-Val-Ala-Pro-Gly-Lys-Ser-Ile-Gly-Gly-Asp-Ile-Phe-Ser-Asn-Arg-Glu-Gly-Lys-Leu-Pro-Gly-Lys-Ser-Gly-Arg-Thr-Trp-Arg-Glu-Ala-Asp-Ile-Asn-Tyr-Thr-Ser-Gly-Phe-Arg-Asn-Ser-Asp-Arg-Ile-Leu-Tyr-Ser-Ser-Asp-Trp-Leu-Ile-Tyr-Lys-Thr-Thr-Asp-His-Tyr-Gln-Thr-Phe-Thr-Lys-Ile-Arg-OH.

Barnase has been covalently bound to agarose (using the cyanogen bromide procedure) in such a manner that ∿65% of molecules are active in binding the inhibitor, barstar. This has made affinity chromatography a practical procedure in the purification of barstar, and some 15-20 mg have indeed been purified with its aid. Rabbit antisera have been prepared against both barnase and barstar, providing a useful tool for studying the complex and for investigating mutant forms of both proteins. (R. W. Hartley).

Protein:Nucleic Acid Interaction: Chromatin Structure and Restriction in Eukaryotic Cells.

During the past year, primary effort has been directed to the understanding of the topographical interrelationships of DNA and proteins in chromatin. Investigation of the geometry and stoichiometry of the binding of a reporter compound [2,4-$(NO_2)_2C_6H_5NH(CH_2)_2N^+(CH_3)_2(CH_2)_3N^+(CH_3)_3 \cdot 2Br^-$.], which is known to bind in the minor groove of the DNA double helix, has indicated qualitatively similar and quantitatively identical binding to native DNA and intact chromatin, demonstrating that the proteins of the chromatin complex could only be bound in the major groove. The minor groove in chromatin is thus left bare, potentially able to interact with any substance capable of recognizing nucleotide

sequences.

Acetylation, under appropriate conditions, modifies only a limited number of lysyl residues in each of the histones of native chromatin, presumably the lysyl groups not involved in the binding of histones to DNA. Native and acetylated chromatin are essentially identical when examined by various physical chemical techniques, and none of the proteins of the nucleoprotein complex are dissociated consequent to the modification. Localization of the acetylated residues in the slightly-lysine-rich histone, FII, indicated that the modified lysyl residues came from an internal section of the molecule, containing relatively few basic, but most of the hydrophobic amino acid residues. These findings suggest that only the terminal portions of the histone are bound to DNA, while the central region either forms a bridge between two portions of a single DNA chain or between two separate DNA chains.

Digestion of chromatin with trypsin cleaves polypeptide bonds in a number equal to the number of lysyl and arginyl residues titratable as "free" (or nonbound) in the nucleoprotein. Thirty percent of the chromatin protein, including peptides derived from all the histones, are thereby dissociated from DNA. The resultant chromatin is nearly identical to the native molecule in terms of thermal denaturation properties, but shows increased intrinsic viscosity and flow dichroism, and has a circular dichroism pattern like that of DNA. Thus, the properties of DNA in chromatin attributed to local charge neutralization are not altered by tryptic removal of the nonbound portions of the histones and nonhistones; while those properties which have been equated with formation of a supercoiled structure for the DNA are altered in the direction of the protein-free nucleic acid by such digestion. These results strongly suggest a bridging role for some of the histones in maintenance of the native conformation of chromatin. (R. T. Simpson).

Fractionation of Chromatin and the Study of Nonhistone Proteins.

A general method for the fractionation of chromatin which avoids the use of acids and ionic detergents in the fractionation procedure has been developed. Chromatin components are separated according to both their molecular weight and their ionic character. Chromatin is dissociated using salt-urea and DNA is spun down by ultracentrifugation, dissociating and separating 90% of the proteins from DNA. These proteins are further fractionated by cation-exchange chromatography, which resolves the distinct histone fractions; while the unretained acidic proteins and RNA are further fractionated on an anion exchanger. Nonhistone components of chromatin have been assigned various roles in protein synthesis, but none of these proteins has been either isolated or characterized. The method developed enables extraction and fractionation of all chromatin components and the isolation of any particular fraction of interest.
(S. Levy, R. W. Simpson, and H. A. Sober).

SECTION ON ENDOCRINOLOGY

Hormonal Induction of Diabetes with Exogenous Hormones.

Diabetes induction in normal rats by growth hormone (GH), ACTH and dexamethasone (Dexa) indicated a strong synergistic diabetogenic effect of GH and

Dexa, with 50% of animals showing ketone bodies at urinary glucose levels of 1/2 - 1 gm/day. In 80% pancreatectomized rats, which rarely excrete ketones, the best synergistic effect appeared with an ACTH-GH combination.

Combination of all 3 hormones induced,in normal rats, such severe ketosis that food intake was reduced and glucosuria could not be used as a diabetes indicator. Measurement of plasma insulin levels established the great ability of the rat's pancreas to produce insulin without becoming exhausted and without depleting its insulin stores. Normal animals given a single 3-hormone dose showed a gradual, several fold increase in plasma insulin level by 16 hours and only a slight rise in blood glucose. Both increased significantly and dramatically within 4 hours after a second injection of the mixture, administered 16 hours after the first, whereas glucose did not appear in the urine until after 48 hours. (R. W. Bates).

Prolactin in Human Blood.

The occurrence in man of prolactin as a protein separate from growth hormone (GH) has been a much disputed point, although there is physiologic evidence in man that the pituitary can secrete one hormone activity without the other. Plasma samples from a patient with a large pituitary tumor, obtained by Dr. Griff T. Ross (NICHD), yielded prolactin levels of 50-70 mμ/ml, 25-50 fold above normal. The ratio of prolactin to growth hormone activity in this case is not compatible with a single protein controlling both activities.
(R. W. Bates).

Diabetic Ketosis

Severe insulin lack, glucocorticoid and growth hormone are necessary for the development of diabetic ketosis. Recent perfusion studies showed that FFA uptake and ketone body production were normal in livers of hypophysectomized rats and that growth hormone and dexamethasone added in vitro were ineffective, indicating that the ketogenic action of these hormones occurs outside the liver. GH and Dexa stimulated FFA mobilization in fasting hypophysectomized rats and this effect was markedly increased when the rats were made diabetic by injection of mannoheptulose. Increased FFA mobilization in these animals resulted from the stimulatory effect of growth hormone on lipolysis and the inhibitory effect of glucocorticoid, in the absence of insulin, or reesterification in adipose tissue.

It can be concluded that the development of ketosis is dependent primarily on the FFA mobilization from adipose tissue to the liver, and that FFA mobilization results from increased lipolysis and decreased reesterification in the adipose tissue. The corrective effect of insulin on ketosis is mediated mainly through its stimulation of reesterification in the adipose tissue.
(S. S. Chernick, C. M. Clark, Jr., R. J. Gardiner & R. O. Scow).

Effect of Hormone on Metabolism of Adipose Tissue Perfused *in vivo*.

Either adrenalectomy or hypophysectomy attenuate the effect of glucagon on FFA release by fat cells *in vitro* and *in vivo*, and these effects are not counteracted by the *in vitro* addition of the missing hormones, indicating the presence in the fat cell of a labile system that requires hormones for its maintenance.

Large doses of ACTH in intact rats may induce increased insulin release, explaining, in part, the peculiar dose response curve of plasma FFA to ACTH injection. Epinephrine treatment, *in vivo*, results in decreased sensitivity of adipose tissue to lipolytic hormones, presumably due to the residual action of insulin secreted in response to the epinephrine injection *in vivo*. These findings emphasize the integrated action of the various hormones *in vivo*. Furthermore, the effects of hormones on blood flow through adipose tissue may play an important role in regulating the growth and metabolism of specific fat bodies in the animal. (V. Kovacev, U. of Skopje, Yugoslavia, and S. Chernick).

Metabolism of Chylomicrons and Lipoprotein Lipase Activity in Adipose and Mammary Tissue.

Studies in perfused adipose tissue showed that more than 90% of the triglyceride taken up is hydrolyzed completely to FFA and glycerol, that 2/3 of the FFA released from chylomicrons are incorporated into tissue triglyceride, and that saturated and unsaturated fatty acids in chylomicrons are similarly metabolized. Heparin infusion caused release of lipoprotein lipase activity to the blood stream without affecting appreciably the total amount of enzyme activity present in the tissue. However, the amount of chylomicrons hydrolyzed by the tissue was reduced 70% both during and after perfusion with heparin, suggesting that the enzyme activity involved in triglyceride uptake is located in or near the capillary bed.

A new electron microscopic technique has been used to determine where chylomicrons are hydrolyzed and how glyceride-fatty acids cross the capillary wall. The findings show that hydrolysis of plasma glycerides by lipoprotein lipase in adipose tissue occurs within capillary endothelial cells and in the subendothelial space (basement membrane area), but not in the capillary lumen. Glyceride-fatty acids are transported across the endothelial cell within a membrane-bounded system, but the chemical nature of the glyceride in transit within the capillary endothelium is not known.

Cytochemical findings clearly show that lipoprotein lipase is present in capillary endothelial cells of adipose tissue. Although fat cells are thought to be the main source of this enzyme, the transfer mechanism to the endothelium has not been determined. The relatively large amount of hydrolysis observed near pericytes in the subendothelial space, suggests that these cells may be also involved in regulation of lipoprotein lipase activity in adipose tissue.

Preliminary results in perfused mammary tissue of lactating rats show that this tissue readily hydrolyzes chylomicron-triglyceride and incorporates the fatty acids into new triglyceride.
(M. Hamosh, J. Blanchette-Mackie, J. Evans, C. Mendelson & R. O. Scow).

Lipolytic Activity in Milk and Tongue

The gastric contents of suckling rats readily hydrolyzes milk lipid to FFA and diglyceride. Although rat milk contains traces of lipoprotein lipase activity, about 0.2% of that in guinea pig milk, it cannot account for the FFA or lipolytic activity found in the stomach. A survey of the lipolytic activity in different tissues showed that the salivary glands, the anterior two-thirds

of the tongue and gastric wall had very little activity whereas the distal third of the tongue contains nearly as much lipolytic activity as pancreas. Studies are in progress to determine whether the enzyme in tongue is involved in the digestion of dietary lipid. (M. Hamosh and R. O. Scow).

MEMBRANE REGULATION SYSTEM

The Adenyl Cyclase System

The adenyl cyclase system in rat parenchymal cells, localized in the plasma membrane, is specifically activated by glucagon and fluoride, each with different activation characteristics. In liver membranes, as in fat cells, the glucagon-sensitive adenyl cyclase system is multimolecular and membrane-bound. Glucagon acts on the system via a "regulatory component" which stimulates the "catalytic component" (adenyl cyclase) to convert ATP to 3',5'-cyclic AMP.

Glucagon incubated with liver membranes results in binding of glucagon without loss of activity as well as an inactivation process. Both processes are functions of membrane concentration, are specific for glucagon, are inhibited or abolished by phospholipases, detergents, urea, trypsin, and are reduced with decreasing temperature of incubation. Binding of glucagon occurs over the identical range of concentrations found for activation of adenyl cyclase by glucagon.

The deleterious effects of phospholipases and detergents on the binding process suggests that the regulatory component is a lipoprotein. Phosphatidylserine restores binding of glucagon to detergent- or phospholipase-treated membranes, suggesting either that phospholipids are involved in binding or are necessary for the integrity of the regulatory component. Hydrophobic forces are implicated in the binding process since glucagon binding is inhibited by low concentrations of urea and by decreasing the temperature of incubation.

Binding of glucagon to the regulatory component was relatively slow. Addition of ATP (>100 μM) or of GTP (50 nM) resulted in a dramatic change in the kinetics of binding, causing maximal binding within 1 minute of incubation and changing the dissociation of bound hormone from an irreversible to a rapidly reversible process. GDP, ADP, and a phosphonate analogue of GTP have the same effects on the kinetics of binding, suggesting that the nucleotides act by binding to site(s), as yet uncharacterized, that alter the properties of the regulatory component. GTP or ATP, at the same concentrations required for altering the binding process, are essential for stimulation of adenyl cyclase by glucagon. It is concluded from these findings that hormonal activation of adenyl cyclase results in a change of the regulatory component from an inactive state (in which binding of glucagon is relatively slow and irreversible) to an active state (in which binding of glucagon is rapid and reversible - a change induced by either GTP or ATP. Furthermore, both the regulatory and catalytic components exist in rapidly reversible states of activity; maintenance of the active states requires the continual presence of both glucagon and GTP (or ATP). Since glucagon and GTP are rapidly destroyed during incubation with liver membranes, the activity of the enzyme, adenyl cyclase, depends upon the free concentrations of these agents, as with isolated plasma membranes.

Structure-Function Relationships in the Glucagon Molecule

Nearly the entire glucagon molecule is essential for function in the adenyl cyclase system. The COOH-terminal region is essential although not uniquely involved in binding of the hormone; the NH_2-terminal histidine is essential for biological action. It is proposed that the COOH-terminal hydrophobic region of glucagon is the initial site of binding of the hormone. The regulatory component behaves as lipoprotein and binds glucagon through forces easily disrupted by agents or conditions that alter hydrophobic interactions. The initial hydrophobic binding induces conformational changes in the remainder of the glucagon molecule, possibly in a hierarchial fashion, that result in a thermodynamically stable complex between glucagon and the regulatory component. The expression of the formed complex is a unique biological activity, the physical chemical basis of which is completely unknown, but which is ultimately reflected by the activation of adenyl cyclase.

(M. Rodbell, L. Birnbaumer, S. Pohl, H. Krans, V. Kozyreff & V. Goldberg).

SECTION ON NUTRITIONAL BIOCHEMISTRY

Metabolism and Function of Fat-soluble Vitamins

In patients with a-Betalipoproteinemia treated with vitamin E, red cell α-tocopherol was normal even though the plasma concentration was one-fourth the normal level. Studies of the dynamics of vitamin E in rat tissues revealed two classes of tissues, those with high storage capacity (plasma, liver, heart and depot fat) and those with low capacity (muscle and testes). When vitamin E was removed from the diet, its concentration in the first class of tissues fell rapidly for two weeks, then decreased slowly as depletion progressed. The second class of tissues showed a slow rate of loss throughout depletion. These studies indicate that plasma vitamin E may not accurately reflect the status of other tissues with respect to the vitamin.

A comparison of the metabolism of α- and γ-tocopherols in rats revealed that α-tocopherol is more efficiently absorbed from the intestine than was γ-tocopherol. The absorption of α-tocopherol was not affected by the degree of unsaturation of the fat administered simultaneously. When injected intraperitoneally, tissue uptake of the two tocopherols was similar, but a more extensive metabolism of γ-tocopherol occurred. Red cells have a slightly greater affinity for γ-tocopherol than for α-tocopherol. (J. G. Bieri, I. R. Peake and H. G. Windmueller)

Biochemical Studies of Cytochromes

Continuing studies of cytochrome c binding to subcellular particles has shown that a minor particle fraction, thought to be immature mitochondria, binds cytochrome c much more tenaciously than do mitochondria or microsomes. The outer mitochondrial membrane has considerably more high affinity binding sites than does the inner membrane. Exogenous cytochrome c was found to penetrate the outer membrane and bind to the inner membrane. Several compounds rich in lysine (poly-L-lysine, poly-L-ornithine) competitively inhibited cytochrome c binding to both mitochondria and microsomes. The inhibitory action of poly-L-lysine on two cytochrome c requiring enzyme systems was

competitive for cytochrome oxidase but uncompetitive for succinate-cytochrome c reductase. (J. N. Williams, Jr.)

Synthesis and Metabolism of Circulating Lipoproteins.

The isolated, perfused rat intestine was found to incorporate radioactive amino acids into the proteins of chylomicrons and low density lipoprotein, which all appeared in lymph, and also into high density lipoprotein which appeared in both lymph and blood. Lipoproteins accounted for half of the newly-synthesized lymph protein. (H. G. Windmueller).

SECTION ON VITAMIN METABOLISM

Studies on Folic Acid

Dihydrofolic reductase is not only a key enzyme in the metabolism of folic acid, it is also of major importance in the synthesis of thymidine and the chief site of action of the anti-folic drugs. Current studies are directed toward further characterization of the various dihydrofolic reductases from yeast and bacteria.

Attempts to use the methotrexate-sepharose affinity column for the purification of dihydrofolic reductase from yeast have been complicated by the presence of unknown substances in yeast which bind to the affinity column more strongly than the enzyme. However, these proteins appear to be of higher molecular weight and can be subsequently removed by gel filtration. Analysis of dihydrofolic reductases from various sources by isoelectric focusing revealed that the enzymes from chicken liver, yeast and leukemia cells exhibit relatively basic isoelectric points whereas the enzymes from bacteria exhibit acidic isoelectric points.

The isolation of the unknown folate found in yeast has been facilitated by a sequential barium precipitation and the use of a DEAE-Sephadex column, resulting in an increased yield and higher purity. If further examination confirms that the complex folate from yeast is pure, attempts to determine its chemical and biological properties will be initiated. (B. T. Kaufman).

Serial No. NIAMD-LNE-1
1. Nutrition and Endocrinology
2. Vitamin Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on folic acid.

Previous Serial Number: SAME

Principal Investigator: Dr. B. T. Kaufman

Other Investigators: Mr. H. A. Bakerman, Miss R. C. Gardiner and Mrs. M. K. Romine

Cooperating Units: None

Man Years

Total:	5
Professional:	4
Other:	1

Project Description:

Objectives: To isolate and determine the biochemical properties of enzymes concerned with the metabolism of folic acid especially dihydrofolic reductase from chicken liver, yeast, bacteria and certain leukemic tumor cells. To study the interaction of these enzymes with agents known to result in activation as well as certain anti-leukemic drugs which result in inactivation. To isolate and determine the chemical and biological properties of the naturally occurring forms of folic acid in various cells and tissues.

Methods Employed: Standard chemical and biochemical techniques appropriate for purification and fractionation of vitamins and proteins are utilized in these projects in conjunction with various methods of enzymatic and microbiological analyses.

Major Findings: Dihydrofolic reductase: (A) Purification: Although the enzyme from yeast is adsorbed to the methotrexate-sepharose column in a manner similar to that previously described for the chicken liver enzyme, the enzyme activity eluted under these conditions appear to be quite impure and unstable. However, significant stabilization occurs after rapid concentration by ultra filtration in the presence of relatively large amounts of dihydrofolate and high salt. Utilizing these conditions, further partial purification could be achieved by means of gel filtration. However, the major question that remains to be answered is why this methotrexate-column binds with such affinity unknown proteins other than dihydrofolic reductase. This unexplained phenomenon not only interferes with the initial binding of the enzyme from the crude extract

but also explains why the product eluted from the column is not homogeneous.

(B) Properties of Dihydrofolic Reductase: The enzymes from various sources have been examined by the electrofocusing technique in which a pH gradient is generated by an applied voltage in a sucrose gradient. The enzymes from chicken liver, yeast and lymphoid leukemia cells exhibited unexpectedly basic isoelectric points; 8.35, 8.57 and 9.41 respectively. Furthermore, two peaks of enzyme activity were observed in each case. The major peak comprising 85-90% of the activity at the previously mentioned basic isoelectric points and the minor second peak at an isoelectric point of approximately 7.6 to 7.9. However, the catalytic properties appear to be identical. The addition of TPHN to the chicken liver enzyme, results in the appearance of a relatively large new peak at pH 6.5 with a concomitant decrease in the size of the pH 8.3 peak and disappearance of the small pH 7.9 peak. On the other hand, the addition of other cofactors such as folate, dihydrofolate, DPNH or TPNH did not affect the original isoelectric profile.

In marked contrast, the dihydrofolic reductase enzymes from bacterial sources, Salmonella typhimurium and Escherichia coli exhibit relatively acidic isoelectric points, 5.45 and 5.25 respectively. In addition, only one peak of enzyme activity is observed with each of these enzymes.

Naturally Occurring Forms of Folic Acid: Although the complex form of folic acid found in yeast has been isolated as a water soluble barium salt the homogeneity of this product is not quite certain. Furthermore, the yields of material obtained by the original procedure are relatively low, i.e., about 10% or 200 mg from 125 lbs of yeast. An improved barium precipitate has decreased the loss of material and the utilization of DEAE-Sephadex rather than DEAE-cellulose at the final stages of purification has increased the chromatographic resolution of the final product.

Significance to Bio-Medical Research and the Program of the Institute: Dihdrofolic reductase is not only a key enzyme in the metabolism of folic acid, it is also of major importance in the synthesis of thymidine and the chief site of action of the anti-folic drugs. Thus, the understanding of the structure and function of this enzyme would be of basic importance in both normal and metabolic interrelationships and cancer chemotherapy. Folic acid as such most probably does not occur in nature. Thus, the knowledge of chemical structure and biological properties of the more complex natural form will result in a better understanding of the biochemistry of this vitamin.

Proposed Course of Project: Investigations will continue on the properties and mechanism of activation of chicken liver dihydrofolic reductase. The lack of similar activations of the enzymes derived from yeast and bacteria will be examined in relation to the properties of this enzyme. This will require the isolation of dihydrofolic reductase from these microbial sources. The current studies on the yeast folate are being directed toward elucidation of its structure and biochemical function.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LNE-2
1. Nutrition and Endocrinology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Large-scale processing of biological materials.

Previous Serial No: Same

Principal Investigator: Mr. D. L. Rogerson, Jr.

Other Investigators: Dr. H. A. Sober

Cooperating Units: None

Man Years

Total:	3
Professional:	1
Other:	2

Project Description

Objectives: Large amounts of natural materials, such as bacteria, animal tissues, cells, blood, urine and plant materials require facilities for their production and processing so that they may be studied under standard laboratory conditions. Facilities for such large-scale processing are provided under this project.

Methods Employed: When called upon for large-scale processing by any NIH investigator, methods are designed for the scaling-up of small laboratory experiments utilizing large reaction vessels, extractors, stills, concentrators, centrifuges, etc., so that the processing can be carried out effectively, efficiently and safely. One 10 l, one 50 l, two 100-gallon and one 300-gallon fermentors are utilized for growing microorganisms under a variety of conditions. The bacterial cells and yeasts are harvested by means of laboratory or large-scale AS-16P centrifuges. Protozoa are concentrated in continuous flow centrifuges. Cells are either supplied as a paste or further processed to yield cell-free suspensions by rupture with a Gaulin Homogenizer. In some instances a specific substance is isolated and partially purified by through processing of the culture supernate or the cell paste. A high capacity, high purification model K Zonal Ultracentrifuge is now installed to handle the increase in production requirements and to provide facilities for gradient centrifugation.

Major Results: During the past year, 298 requests by NIH investigators were processed. 165 kilogram quantities (52,000 liters of cultures) of 17 microorganisms were produced. The microorganisms grown during this period included protozoa, yeast and bacteria and consisted of regular and mutant strains

of *Acanthamoeba castellanii, Aerobacter cloacae, Bacillus amyloliquefaciens, Bacillus subtilis, Clostridium thermosaccharolyticum, Comamonas sp., Escherichia coli, Hemophilus influenzae, Methanococcus vannielii, Paramecium aurelia, Pseudomonas acidivorans, Pseudomonas purtida, Pseudomonas sp., Pseudomonad GS, Saccharomyces cerevisiae, Salmonella typhimurium and Alpha streptococcus.* Some of the organisms were used as hosts for bacteriophage production such as Q beta, Lambda, MS-2, T_4, and T_7 and others, such as *Bacillus amyloliquefaciens* and mutant strains of *Escherichia coli* were used to produce extracellular enzymes. Microorganisms were grown 125 times in the 300 and 1100 liter fermentors, 44 times in the 10 liter New Brunswick fermentor which includes 10 times as starter inocula for the large fermentors and 15 times in the new 50 liter Fermentation Design fermentor. Optimum growth conditions for maximum polysaccharide production by *Hemophilus influenzae* were studied during eleven fermentations from 10 - 300 l. for the NICHD. Also two 50 l. cultures of *Escherichia coli* were processed for cross-reacting polysaccharide and a simplified method for large scale production of a heat-sensitive medium component was investigated. Hepatoma tissue cells were grown successfully once in the 10 liter fermentor and twice in the 50 liter fermentor, and efforts were made to recover the costly medium aseptically during separation and recovery of the cells in the continuous flow system of the Sorval RC-2B centrifuge. The Gaulin homogenizer was used 88 times to rupture suspensions of yeast or bacterial cells. Large-scale processing activities consisted of isolation of cell nuclei from 8 kgm fresh calves liver, Hurler Factor from 34 liter human, male urine, histamine methylating enzyme from 1.5 kgm guinea pig brains, special inhibitor from 30 kgm *Bacillus amyloliquefaciens*, aminotransferase from 13.4 kgm *Salmonella typhimurium*, EXO-3 nuclease from 2 kgm *Escherichia coli B*, polynucleotide phosphorylase from 1 kgm dry *Micrococcus lysodeiketicus* and partial processing of 200 lbs Baker's yeast for folates. The large capacity and turbine Sharples centrifuges were used to separate cells from cultures such as 190 liter *Mycoplasma hyorhinis* and 23 liter *Hydrogenomonas utropha*. Hydroxylapatite was prepared four times in the 50 gallon Pfaudler reactor yielding 8 gallons of the chromatographic adsorbents and 56 liters of Leventhal base was prepared from horse blood. 150 lbs of animal diet and various quantities of seeds and resins were ground in the laboratory mills. Ether and alcohol extracts of plant material were concentrated in vacuo in the explosion-proof area and 140 liters of radioactive sweat water were concentrated below room temperatures in the high capacity vacuum system.

Significance to Bio-Medical Research and the Program of the Institute: With the laboratory's facilities many projects can be assisted where large a amounts of natural material be produced and processed.

Proposed Course of Project: To continue assisting NIH investigators when large-scale processing of biological materials is needed in their projects and to expand the facilities for isolation and purification of biologically-important macromolecules, cell particulates and materials.

Honors and Awards: None

Publications:

Rushizky, G. W., Mozejko, J. H., Rogerson, D. L. Jr., and Sober, H. A.: Characterization of enzymatic specificity of a RNase from *Ustilago sphaerogena* Biochemistry 9: 4966-4970, 1970.

Serial No. NIAMD-LNE-3
1. Nutrition and Endocrinology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in experimental nutrition.

Previous Serial Number: Same

Principal Investigator: Mr. E. G. McDaniel

Other Investigators: Dr. F. S. Doft, Consultant, OD

Cooperating Units: Dr. G. Laqueur, M. Spatz (LEP-NIAMD)
Dr. R. L. Vought (EFS-NIAMD)
Dr. J. Bieri (LNE-NIAMD)
Dr. W. Rogers, NIDR
Dr. J. Smith, Veterans Admin. Hosp., Trace Element Lab.

Man Years

Total:	3
Professional:	1
Other:	2

Project Description:

Objectives: To determine the nutritional, biochemical and physiological characteristics of germfree and conventional laboratory animals.

Methods Employed: Germfree and conventional rats, and chicks are maintained on diets of known composition and growth, blood picture, and other physiological functions are studied. Germfree animals are inoculated with known strains of bacterium to determine the organisms responsible for various phenomena. Conventional and/or ex-germfree animals are used in conjunction with germfree work.

Major Findings: With Dr. Laqueur and Dr. Spatz, studies of the toxic effects of cycasin have been continued. Cycasin is not toxic to adult germfree rats but is toxic to conventional rats, very young germfree rats and to previously germfree rats infected with bacteria which are capable of converting the compound to its aglycone. Previously germfree rats, infected with bacteria which are unable to make this conversion, react like germfree rats and show no symptoms of toxicity to cycasin. The aglycone of cycasin is toxic to germfree as well as conventional rats at any age. Although adult rats are incapable of converting cycasin to the aglycone in the absence of bacteria, the very young animal is able to do so but this capability is lost early in life. Experiments in progress are designed to determine at what period the

animal loses the ability to make this conversion within its own tissue and without the presence of bacteria. Single doses of cycasin were administered to germfree rats at five, nine,sixteen, twenty-five and thirty-five days of age. At the present time tumors have been observed after several months in animals which received cycasin as late as twenty-five days of age. It is too early to determine whether later administration will be effective.

With Dr. Vought, experiments have been continued to study the effects of bacteria of "germfreeness" upon iodine metabolism, thyroid function and the development of goiter. Methods studied to effect an alteration of the intestinal flora of rats include use of antibiotics, monoinfection or controlled infection of previously germfree rats with various types of bacteria, "over-infection" of conventional animals and isolation. The effects of administration of sterile preparations of toxins of bacterial origin are also being studied. Changes observed appear to be related to the magnitude of the infection as well as to the type of organism used.

With Dr. Bieri and Dr. Rogers, the study of vitamin A deficiency in rats and chickens is being continued. Germfree rats are able to survive for long periods without demonstrable amounts of vitamin A compared to conventional and ex-germfree rats fed similar diets. Conventional rats develop the typical symtpoms of vitamin A deficiency and stop growing at about six weeks, then lose weight rapidly and die. Although germfree rats also reach a weight plateau and develop the typical symptoms of vitamin A deficiency they continue to survive for periods up to a year. It has been observed that if some of the dietary ingredients (cornstarch and casein) were preheated in air in a dry oven at 100° C. before preparation of the diet, there was a significant decrease in the survival time of the germfree rats. This suggests that during the preheating, trace amounts of vitamin A may be destroyed by oxidation. That this is probably true is suggested by the observation that administration of vitamin A restored growth to normal and corrected the deficiency symptoms. The decrease in survival time of germfree rats fed the heated diet suggests that the difference noted between germfree and conventional rats may result from differences in the efficiency with which germfree and conventional rats are able to utilize very small amounts of vitamin A, and not to the lack of a requirement of this vitamin for survival. To determine whether the early death in conventional rats may be due to infection rather than the A deficiency itself, vitamin A deficient germfree rats were separated into two equal groups. One group was maintained germfree and the other was infected with a pure culture of *E. coli*. The presence of this organism appeared to have no effect upon the animals. The same group was later infected with the bacterial flora from an A-deficient conventional rat. This was followed by an immediate and rapid weight loss then death in less than two weeks. Vitamin A supplemented rats which were likewise infected and maintained in the same isolator showed no deleterious effects of the bacteria. The group which was maintained germfree survived and have maintained the weight plateau. These results indicate that the early death of conventional animals is the result of infection rather than the lack of vitamin A itself. It is further suggested that the deaths are caused by specific organisms and not by the presence of just any

bacteria. Studies with chickens indicate that germfreeness does not afford protection against vitamin A deficiency, either with respect to growth, survival or onset or severity of the symptoms, with this species as it does with the rat. Experiments are in progress to study the actual lesion caused by vitamin A deficiency, and the metabolic function of vitamin A at the cellular level. This may be feasible since it is possible to keep germfree animals alive in a state of very severe vitamin A deficiency for long periods.

With Dr. Smith, a series of experiments have been conducted to determine whether the requirement for, or the deficiency symptoms of the trace element zinc are effected by the intestinal bacteria. Results to date have shown that bacteria can play an important role in the development of symptoms and survival time of rats fed zinc deficient diets. There is indication that the early death observed in conventional zinc-deficient rats may be due in part to infection. It has been possible to keep germfree rats alive for long periods on low zinc diets which result in early death in conventional rats. It was further observed that the protection afforded by germfreeness was not the same under all conditions. It appears that as lower and lower levels of dietary zinc have been achieved the difference between germfree and conventional rats becomes less. This may indicate that the differences observed could be the result of a more efficient utilization of very small amounts of dietary zinc in the absence of a bacterial flora. It was further observed that the utilization of small amounts of zinc can be markedly influenced by the presence or absence of other minerals in the diet. Studies are in progress to study these interrelationships in detail. The poor growth which has been observed with rats fed autoclaved diets containing egg white was found to be in part due to an imbalance of amino acids. The poor growth could be partially overcome by addition of certain amino acids. Threonine appeared to be the amino acid most effected. It was found that egg white diets sterilized by irradiation with a cobalt source permitted growth which compared favorably with that of raw egg white, which is very low in zinc. Therefore, it should be possible to use egg white as a source of protein for germfree studies. The work has been hampered somewhat by the occurrence of kidney stones in germfree rats on low zinc diets. Progress has been made in overcoming this difficulty but further work is being done.

With Dr. Doft, experiments are being continued to determine methods of altering the intestinal flora of animals in such a way as to have a beneficial effect upon a host which is subjected to diets of limited nutritional quality. It has been found with conventional animals that combinations of certain antibiotics or isolation along with antibiotics (life-island) it is possible to maintain a bacterial flora in rats which does permit better utilization of inadequate diets. This provides basic information in planning human studies in an area where infant diseases of malnutrition occur.

Significance to Bio-Medical Research and the Program of the Institute:
The work with germfree animals will give us a better understanding of the relations between the animal's intestinal flora and its physiological reactions both to dietary nutrients and to other factors. The work with the germfree animals emphasizes the importance of nutrition in long-term studies.

Proposed Course of Project: Experiments will be conducted in an attempt to determine more precisely the relationship between the host animal and its bacterial flora with respect to its influence upon the nutrition and physiology of the host. Attempts will be made to identify the specific organisms which are responsible for such influence and, if possible, determine methods to counteract such influences as may be deleterious to the host.

Honors and Awards: None

Publications:

Vought, R. L., Brown, F. A., Sibinovic, K. H. and McDaniel, E. G.: Effect of changing intestinal bacterial flora on thyroid function in the rat. Hormone and Metabolism Research. In press.

Serial No. NIAMD-LNE-4
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Study of protein hormones: Chemistry and physiological actions of protein hormones.

Previous Serial Number: NIAMD-LNE-4

Principal Investigator: Dr. Robert W. Bates

Other Investigators: None

Cooperating Units: None

Man Years

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: To isolate protein hormones from the pituitary glands of man and animals, functional, transplantable mouse or rat pituitary tumors, and blood, especially the anterior pituitary hormones; to investigate the nature of these hormones as they circulate in the bloodstream; to correlate chemical structure and biological function; to study the effect of hormones produced in excess by tumors on the host animal; to investigate the induction of diabetes by hormones.

Methods Employed: Hormones and other substances are extracted from pituitary gland, rat or mouse pituitary tumors, pancreas and blood. Crude extracts which may contain several hormones are fractionated by techniques commonly used in protein chemistry, such as salt precipitation, solvent fractionation, iso-electric precipitation, preparative and disc electrophoresis, ion-exchange chromatography, and gel filtration. Various quantitative biological assays are required to determine the concentration of the several hormones in tissues and protein fractions. Radioimmunoassay techniques are employed to extend the studies to concentration levels beyond the reach of conventional bioassay procedures. Removal of various endocrine glands with and without hormonal replacement is a critical technique. Effects of elimination or modification of hormonal production of the thyroid by anti-thyroid drugs and of the adrenal cortex by anti-adrenal drugs is compared to effects of surgical removal of the gland.

Major Findings: Hormonal Induction of Diabetes with Exogenous Hormones: The induction of diabetes by exogenous hormones in normal rats with intact pancreas has been studied and compared with that in 80% pancreatectomized rats (80%$\overline{P}$). Growth hormone (GH), adrenocorticotropin (ACTH) and dexamethasone (Dexa) were given singly or in various combinations at different dosage levels. The minimum effective dose (MED) of dexamethasone needed to induce glucosuria in normal rats, with intact pancreas, was 5 times that in 80%$\overline{P}$ rats, whereas GH and ACTH given individually at 5x MED gave negative results. Higher dosages were not practical.

A strong multiplicative (synergistic) diabetogenic effect was found with combinations of GH and Dexa in intact rats. About half of the rats had ketone bodies in their urine as soon as there was 0.5-1.0 g of glucose in the urine per day. This finding is unlike that in 80%$\overline{P}$ rats, which rarely excrete ketones. In normal rats combinations of ACTH and Dexa or ACTH and GH were much less synergistic, if synergistic at all. In 80%$\overline{P}$ rats, however, the ACTH and GH combination gave the best multiplicative effect.

Combined injections of all three hormones showed a synergistic action in normal rats but the diabetogenic effect was not much greater than that obtained from the combination of GH and Dexa. Ketosis occurred to such an extent that food intake was greatly reduced and hence interfered with the use of glucosuria as a measure of the degree of diabetes.

All rats were killed on the fifth day for determination of insulin concentrations in plasma and pancreas. The blood sugar level was generally found to be correlated with glucosuria but in cases of ketosis it tended to be higher. Plasma insulin levels were raised 10 fold from a normal level of 30-50 U/ml to 200-500 μU/ml with effective diabetogenic doses. At the same time the insulin concentration in the pancreas was rarely changed significantly. This indicates the great ability of the rat's pancreas to produce insulin without depletion of its insulin stores or becoming exhausted.

A study of the time course of these increases in plasma glucose and insulin in normal animals after a single injection of a mixture of the three hormones showed a gradual increase in plasma insulin level which was several fold by 16 hours; the increase was statistically significant at 8 hours. At the same time the blood glucose level rose slightly but not significantly. Both increased significantly and dramatically within 4 hours after a second injection of the mixture 16 hours subsequent to the first injection. Glucose does not appear in the urine until about 48 hours even at higher dosage.

Prolactin in Human Blood: The occurrence in man of prolactin as a protein separate from growth hormone (GH) has been a much disputed point. Acromegaly without mammary stimulation, and galactorrhea, gynecomastia and normal lactation without acromegaly indicate that the pituitary can secrete one hormone without the other. The local cropsac bioassay in the pigeon is not sensitive enough to detect prolactin activity in extracts of normal blood. The positive cropsac responses obtained with extracts of plasma from patients with galactorrhea and gynecomastia, and from patients a few days postpartum are

questionable due to concomitant inflammatory reactions. Dr. Griff Ross (NCI) supplied us with 8 plasma samples taken before and after glucose, insulin and arginine tolerance tests and during Piromen tests on a patient, D. C., who had a large pituitary tumor. After extractions all eight plasma samples had a prolactin concentration of 50-70 mu/ml, which is about 4 times greater than that observed in patients with galactorrhea or postpartum. Also the responses were not questionable and were unhindered by the inflammatory reaction. Tests by others showed that the growth hormone level in these plasma samples were normal whereas we found that the prolactin levels were elevated 25 to 50 fold, at least, above normal. Although pure GH as isolated from human pituitary glands has some inherent prolactin activity, the ratio of prolactin activity to GH activity in the plasma of patient D. C. is not compatible with the concept of a single protein, GH, controling both growth and lactogenic activity. A separate prolactin molecule probably exists in man, as in all other nonprimate mammalian species tested.

Significance to Bio-medical Research and the Program of the Institute: One of the major roles of the six hormones of the anterior pituitary gland is to modify or regulate the size and functional activity of other glands or tissues, which in turn control the overall metabolic state of the individual. This hormonal balance or imbalance controls how one feels and what one can do. Certain metabolic abnormalities and tumors are corrected by treatment with hormones or by removal of endocrine glands. Purified hormone preparations are needed for this work together with sensitive and quantitative tests which will estimate the concentration of hormones in the blood or transport system and the rate of release of the hormones. One needs to know what factors control the biosynthesis and subsequent release of hormones by the pituitary gland.

The studies on induction of diabetes reveal the major role of interaction in a synergistic manner of at least three hormones or types of hormones. The extent of this synergism is such that hormonal induction of diabetes in man should be seriously investigated.

Proposed Course: Studies on the induction of diabetes by hormones in rats that are 80% pancreatectomized will be carried out in a systematic manner to throw light on the problem of hormonal interactions (potentiation or inhibition) and to assess the significance of hormonal balance.

Analysis of hormone levels in rats with transplantable pituitary tumors will continue. A search for factors which modify these levels will be made. The physiological action of the hormones from the pituitary tumors upon the host will be studied. Studies on the changes occurring after tumor removal are planned.

The question of identity of human GH and prolactin results in confusion when one attempts to establish potency of prolactin in plasma of humans in various clinical conditions. Also the specificity of the cropsac response in pigeons raises the question of whether the response obtained with urine and plasma extracts is due to prolactin. These problems are being investigated.

Honors and Awards: None

Publications:

Bates, Robert W. and Garrison, Mary M. Solid-phase Radioimmunoassay of Insulin. In: Sunderman, F. W. and Sunderman, F. W. Jr. (Editors) Laboratory Diagnosis of Endocrine Disorders, Warren H. Green, Inc., St. Louis, 1971, p. 332.

Serial No. NIAMD-LNE-5
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Diabetes and Fat Metabolism

Previous Serial Number: NIAMD-LNE-4

Principal Investigators: Drs. Robert O. Scow and Sidney S. Chernick

Other Investigators: Dr. Joan Blanchette-Mackie, Staff Associate
Dr. Margit Hamosh, Visiting Scientist
Dr. Carole Mendelson, Guest Worker
Dr. Anthony J. Evans, Guest Worker

Cooperating Units: None

Man Years:

Total:	9
Professional:	4
Others:	5

Project Description:

Objectives: To determine influence of hormones and other factors on metabolism of fat and carbohydrate in liver, adipose tissue and mammary gland of normal and diabetic animals.

Methods Employed: Animals are deprived of one or several endocrine glands and treated with hormones or other substances. The effect of the above on metabolism of fat and carbohydrate in liver, adipose tissue and other organs and on blood hormone levels are determined with in vivo and in vitro studies using conventional and radioisotope techniques, and electron microscopy. Effects of hormones added in vitro are also studied.

Major Findings: Diabetic Ketosis: Previous studies in this laboratory showed that severe insulin lack, glucocorticoid and either growth hormone, ACTH or thyroxine are necessary for the development of diabetic ketosis. Ketosis is characterized by abnormally high concentrations of β-OH-butyrate and acetoacetate in plasma and urine, and is always accompanied by a high concentration of free fatty acids (FFA) in the plasma. Perfusion studies, made in this laboratory and elsewhere, have established that ketone body production by the liver is directly related to the concentration of FFA in the plasma. Recent experiments in fasting rats showed that the rates of turnover of FFA and ketone bodies in plasma are both increased 100% by diabetes ('total' pancreatectomy). The relative rates of turnover of FFA and

ketone bodies in the diabetic rats, and also in the normal, suggest that about 50% of the FFA mobilized are partially oxidized to ketone bodies in the liver before they are utilized by other tissues. Experiments reported last year showed that uptake of FFA and ketone body production were normal in perfused liver of rats that had been hypophysectomized for more than 2 weeks. Recent studies showed that growth hormone and dexamethasone, added to the perfusing fluid, have no effect on either FFA uptake or ketogenesis in perfused livers of hypophysectomized rats. These findings indicate that the ketogenic action of these hormones does not occur in the liver.

Growth hormone and dexamethasone given together stimulate lipolysis (isolated fat cells, 1965) and FFA release (in vivo perfusion of adipose tissue, 1965) in fasted normal rats. Their effect on lipid mobilization has now been studied in fasting hypophysectomized rats made diabetic by injection of mannoheptulose. The latter given alone increased within 4 h the plasma concentrations of glucose and glycerol 100%, and that of FFA and ketone bodies 50%. Growth hormone injected with mannoheptulose increased further the concentrations of plasma FFA and ketone bodies but not that of glucose nor glycerol. Dexamethasone augmented the effect of mannoheptulose on plasma glucose 100% and on FFA 30%, but not on glycerol nor ketone bodies. Growth hormone and dexamethasone given together increased considerably the effect of mannoheptulose on all four plasma constituents. Incubation studies of adipose tissue taken 4 hours after injection of mannoheptulose and hormones showed that the rates of lipolysis and FFA release were highest and glucose utilization was lowest in tissues of rats given mannoheptulose and both hormones.

It is concluded from these and earlier studies that the development of ketosis is dependent primarily on the mobilization of FFA from adipose tissue to the liver, and that FFA mobilization results from increased lipolysis and decreased reesterification in the adipose tissue. In the uncontrolled diabetic lipolysis is stimulated by a pituitary factor, probably growth hormone, and reesterification is suppressed by the inhibitory effect of glucocorticoid on glucose utilization in the absence of insulin. The corrective effect of insulin on ketosis is mediated mainly through its stimulation of glucose utilization and reesterification in the adipose tissue.

Metabolism of chylomicrons and lipoprotein lipase activity in adipose and mammary tissue: There is considerable evidence now that uptake of blood triglyceride-fatty acids by most tissues (adipose, mammary and muscle) involves hydrolysis of the triglyceride to FFA and that hydrolysis is catalyzed and regulated by lipoprotein lipase. These processes are being studied with biochemical and cytochemical techniques in perfused adipose and mammary tissue of the rat.

Studies made recently in perfused adipose tissue, using doubly labeled chylomicrons (glyceride-^{3}H-glycerol and ^{14}C-palmitic acid), showed that more than 90% of the triglyceride taken up is hydrolyzed completely to FFA and glycerol, and that 2/3 of the FFA released from chylomicrons are incorporated into tissue triglyceride. Experiments using triply labeled chylomicrons

(glyceride-^{3}H-glycerol, ^{3}H-oleic acid and ^{14}C-palmitic acid) showed that saturated and unsaturated fatty acids in chylomicrons are similarly metabolized by the tissue. Heparin infusion caused release of lipoprotein lipase activity to the blood stream without affecting appreciably the total amount of enzyme activity present in the tissue. However, the amount of chylomicrons hydrolyzed by the tissue was reduced 70% both during and after perfusion with heparin, suggesting that the enzyme activity involved in triglyceride uptake is located in or near the capillary bed.

A preliminary description was given last year of a new electron microscopic cytochemical technique for studying lipoprotein lipase activity in tissue. This technique has been refined and used to determine where chylomicrons are hydrolyzed and how glyceride-fatty acids cross the capillary wall in adipose tissue. The tissues are perfused with chylomicrons, fixed in cold glutaraldehyde, incubated in $CaCl_2$-Tris medium (pH 8.3) at 38°C, and then treated with $Pb(NO_3)_2$ solution. Granular and laminar electron-dense precipitates, made by the reaction of Pb^{++} with the insoluble fatty acid soaps formed during incubation, are deposited at the sites of hydrolysis. The findings show that hydrolysis of plasma glycerides by lipoprotein lipase in adipose tissue occurs within capillary endothelial cells and in the subendothelial space (basement membrane area), but not in the capillary lumen. The results also indicate that glyceride-fatty acids are transported across the endothelial cell within a membrane-bounded system. The chemical nature of the glyceride in transit within the capillary endothelium, however, is not known.

The cytochemical findings clearly show that lipoprotein lipase is present in capillary endothelial cells of adipose tissue. Although fat cells are thought to be the main source of this enzyme in adipose tissue, the manner in which the enzyme could be transferred to the endothelium has not been determined. The relatively large amount of hydrolysis observed near pericytes, in the subendothelial space, suggests that these cells may be also involved in regulation of lipoprotein lipase activity in adipose tissue.

The studies in perfused rat mammary tissue are still preliminary. A procedure has been developed for perfusing in situ the abdominal-inguinal mammary glands on one side, via a cannula inserted into the superficial epigastric artery, a branch of the femoral artery, and collecting venous blood from the superficial epigastric vein. The other vessels serving the tissue are occluded by ligation. Preliminary results show that mammary tissue of lactating rats readily hydrolyzes chylomicron-triglyceride and incorporate the fatty acids into new triglyceride.

Lipolytic activity in milk and tongue: It was reported last year that 10% of the lipid in the gastric contents of suckling rats is present as FFA, whereas less than 1% of the lipid in fresh milk is FFA. Lipolytic activity was also found in the gastric content. Based on the high lipoprotein lipase activity found in guinea pig milk, it was suggested at that time that the lipolytic activity found in the stomach contents of suckling rats might be

due to enzyme secreted in the milk. Study of the lipolytic activity in rat and guinea pig milk showed that the activity in both milks was due to lipoprotein lipase, but the activity in rat milk was very low, less than 0.2% of that in guinea pig milk, and probably could not account for that found in the gastric contents. A survey was then made in suckling rats of the lipolytic activity in different tissues associated with digestion. It was found that the salivary glands and anterior two-thirds of the tongue had very little if any activity, whereas, the distal third of the tongue had nearly as much lipolytic activity as pancreas. The enzyme activity in the tongue hydrolyzes triglyceride to diglyceride and has a pH optimal between 4.9 and 5.7. Studies are in progress to determine whether this enzyme is secreted into the buccal cavity, and if so, its role in digestion of dietary lipid.

Significance to Biomedical Research in the Program of the Institute: Free fatty acids are mobilized from adipose tissue during fasting and in uncontrolled diabetes in order to provide energy for other tissues in the body. The studies described above indicate that in both normal and diabetic individuals about half of the FFA mobilized are first partially oxidized to ketone bodies in the liver before they are utilized by the other tissues. The studies also demonstrate that adipose tissue is the primary site of action of the hormonal factors - insulin-lack, glucocorticoid and growth hormone - that cause diabetic ketosis. Ketone bodies are produced at an accelerated rate by the liver whenever the plasma FFA concentration is increased. Insulin probably corrects ketosis by suppressing FFA release from adipose tissue, through its stimulatory effect on glucose utilization and reesterification.

The studies of lipolytic activity in the tongue suggests that this tissue may contribute to the digestion of dietary lipid, perhaps by hydrolyzing triglyceride to partial glycerides in the stomach before the lipid enters the intestines. This interesting possibility requires further study.

It has often been suggested that blood triglycerides may be hydrolyzed near or in the capillary wall when they are removed from the circulation. The electron microscopic cytochemical study described above has now shown that blood triglycerides are hydrolyzed within the capillary endothelium and in the subendothelial space of adipose tissue, but not in the lumen. The study also shows that lipoprotein lipase is present in endothelial cells, and perhaps also in pericytes. The results show that glyceride-fatty acids are transported across the capillary endothelium within a membrane-bounded system. Whether a similar process occurs in the endothelium of other vessels should be studied.

Proposed Course: Hormonal and nutritional factors involved in the conversion of plasma FFA to CO_2, ketone bodies, tissue triglyceride and very low density lipoproteins in livers of fasting and diabetic animals will be studied with biochemical and morphological techniques.

Study of the effect of hormones and other factors on lipoprotein lipase activity in mammary and adipose tissue will be continued. The site of

formation and action of the enzyme, as well as mechanism of its action, and the role of capillary endothelium in the hydrolysis of blood triglyceride will be investigated.

The effects of hormones on structure and function in adipose tissue, liver, mammary gland and blood capillaries will be studied by electron microscopy. The role of the capillary endothelium in transfer of hormones, nutrients and enzymes between the blood and tissue cells will also be studied.

Honors and Awards: None

Publications:

Bieberdorf, Frederick A., Chernick, Sidney S. and Scow, Robert O. Effect of insulin and acute diabetes on plasma FFA and ketone bodies in the fasting rat. J. Clin. Inv., 49:1685-1693, 1970.

Blanchette Mackie, E.J. and Scow, R.O. Sites of lipoprotein lipase activity in adipose tissue perfused with chylomicrons: electron microscopic cytochemical study. J. Cell Biol. In Press.

Chernick, Sidney and Novak, Milan. Effect of insulin on FFA mobilization and ketosis in fasting pregnant rats. Diabetes, 19:563-571, 1970.

Clark, Charles M. Jr. and Scow, Robert O. Effects of fasting and hypophysectomy on FFA uptake and ketone body production by the isolated, perfused rat liver. Diabetes, 19:924-929, 1970.

Hamosh, M. and Scow, R. O. Plasma triglyceride and mammary and adipose tissue lipoprotein lipase in pregnant and lactating rats. Iugoslav. Physiol. Pharmacol. Acta, 6:169-173, 1970.

Hamosh, Margit, Clary, Theresa R., Chernick, Sidney S. and Scow, Robert O. Lipoprotein lipase activity of adipose and mammary tissue and plasma triglyceride in pregnant and lactating rats. Biochim. Biophys. Acta, 210:473-482, 1970.

Hamosh, M. and Scow, R.O., Lipoprotein lipase activity in guinea pig and rat milk. Biochim. Biophys. Acta, 231:283-289, 1970.

Stein, Olga, Scow, Robert O. and Stein, Yechezkiel. FFA-^{3}H uptake by perfused adipose tissue: electron microscopic autoradiographic study. Am. J. Physiol., 219:510-518, 1970.

Serial No. NIAMD-LNE-6
1. Nutrition and Endocrinology
2. Membrane Regulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanism of Action of Hormones on Plasma Membranes of Liver and Adipose Cells

Previous Serial Number: NIAMD-LNE-6

Principal Investigator: Dr. Martin Rodbell

Other Investigators: Dr. Lutz Birnbaumer, Staff Fellow
Dr. Stephen L. Pohl, Staff Associate
Dr. H. Michiel Krans, Visiting Scientist
Dr. Vladimir Kozyreff, Visiting Scientist
Mrs. Vivian Goldberg, Guest Worker

Cooperating Units: None

Man Years

Total:	4
Professional:	3
Other:	1

Project Description:

Objectives: To determine the mechanism of action of various peptide hormones on adenyl cyclase systems in plasma membranes of liver and adipose cells.

Methods Employed: Sacs of plasma membranes, termed ghosts, are isolated from fat cells of the rat after hypotonic treatment and differential centrifugation. Fragments of plasma membranes of hepatic parenchymal cell origin are isolated from rat liver after gentle homogenization of liver, followed by a series of centrifugations in discontinuous and continuous gradients of sucrose. Adenyl cyclase activity and binding of labeled glucagon were carried out by procedures developed in this laboratory. Derivatives of glucagon were prepared by Edman degradation, trypsin digestion, cyanogen bromide treatment, or by chemical synthesis.

Major Findings: The adenyl cyclase system in rat parenchymal cells is localized in the plasma membrane and is specifically activated by glucagon. Fluoride ion also activates the enzyme but, as previously found for the fat cell enzyme system, by a process that has different characteristics from the hormone-stimulating process. Hormonal activation is inhibited or abolished

after treatment of plasma membranes with phospholipases, detergents, or low (0.4M) concentrations of urea, and is enhanced by chelating agents such as EDTA. The fluoride-activation process is markedly inhibited by pyrophosphate, is enhanced by Mn^{++}, and is either enhanced or unaffected by phospholipases, detergents, and chelators. The enzyme system is inhibited by sulfhydryl agents and by calcium ion in the presence or absence of glucagon or fluoride ion. It is concluded that the glucagon-sensitive adenyl cyclase system in liver membranes, as has been previously found with fat cells, is a multimolecular, membrane-bound system and that glucagon acts on the system through a separate component, termed "regulatory component", from the enzyme or "catalytic component" that converts ATP to 3'5' cyclic AMP.

Reactions of glucagon with "regulatory component": ^{125}I-glucagon was used to determine the fate of glucagon incubated with liver membranes and the characteristics of the reaction between the hormone and the regulatory component. Two reactions were observed. One involved binding of glucagon by forces readily dissociable with urea and which did not result in modification of the biological activity of the hormone. The other process involved inactivation of the hormone to give a product, still uncharacterized, which no longer binds to liver membranes or activates adenyl cyclase. Both the binding and the inactivating processes share similar characteristics. Both processes are functions of membrane concentration, are specific for glucagon, are inhibited or abolished by phospholipases, detergents, urea, trypsin, and are reduced with decreasing temperature of incubation. Binding of glucagon occurred over the identical range of concentrations found for activation of adenyl cyclase by glucagon.

The essentially identical characteristics displayed by both the binding process and the hormone activating process suggests that the site of binding represents the regulatory component. The structural and functional relationship of the inactivating process to the adenyl cyclase system requires further investigation but seems to share many of the properties exhibited by the regulatory component.

The deleterious effects of phospholipases and detergents on the binding process suggests that the regulatory component is a lipoprotein. Phosphatidylserine, among various lipids tested, restores binding of glucagon to detergent- or phospholipase-treated membranes suggesting either that phospholipids are involved in binding or are necessary for structure of the regulatory component. Hydrophobic forces are implicated in the binding process since glucagon binding is inhibited by low concentrations of urea and by decreasing the temperature of incubation.

Binding of glucagon to the regulatory component was relatively slow; maximal binding occurred only after about 15 minutes when membranes were incubated in buffered albumin. Under these conditions, binding was essentially irreversible. Addition of ATP, the substrate for adenyl cyclase, at concentrations greater than 100 μM, or of GTP at concentrations as low as 50 nM, resulted in a dramatic change in the kinetics of binding. The nucleotides caused maximal binding to be achieved within 1 minute of incubation and

changed dissociation of bound hormone from an irreversible to a rapidly reversible process. In the presence of 1 μM GTP, bound glucagon completely dissociates within 2 minutes of incubation. GDP, ADP, and a phosphonate analogue of GTP have the same effects on the kinetics of binding, suggesting that the nucleotides act by binding to site(s), as yet uncharacterized, that alter the properties of the regulatory component.

Role of Nucleotides in Glucagon Action: GTP or ATP, at the same concentrations required for altering the binding process, are essential for stimulation of adenyl cyclase by glucagon. In the absence of the nucleotides, even supra-maximal concentrations of glucagon fail to stimulate adenyl cyclase. It is concluded from these findings that hormonal activation of adenyl cyclase involves changing the regulatory component from an inactive state (to which binding of glucagon is relatively slow and irreversible) to an active state (to which binding of glucagon is rapid and reversible). The change in states is induced by either GTP or ATP.

Since binding of glucagon is essential for activation of adenyl cyclase and is only transient when the regulatory component is in its active state (GTP or ATP induced), activation of the catalytic component should only occur during the brief moment of occupation of the regulatory component and should then rapidly decay either when the hormone is withdrawn or when the regulatory component becomes occupied by an inactive analogue of glucagon. It was found that dilution of the hormone concentration at any time during incubation (with GTP or ATP) resulted in immediate decay of activity of the enzyme to that predicted by the diluted concentration of hormone. Addition of des-histidine glucagon, a bindable but non-active form of glucagon (see below), at any time during activation of the enzyme by glucagon resulted in decay of activity to basal (non-stimulated) activity within 45 seconds of addition of the analogue. It is concluded from these findings that both the regulatory and catalytic components exist in rapidly reversible states of activity; maintenance of the active states requires continual presence of both glucagon and of GTP (or ATP). Since glucagon and GTP are rapidly destroyed during incubation with liver membranes, the moment to moment activity of the enzyme depends upon the free concentrations of these agents, as is observed with isolated plasma membranes.

Structure-function Relationships in the Glucagon Molecule: Removal of the NH_2-terminal histidine residue by a one-step Edman degradation of glucagon results in loss of biological activity of the hormone as assessed by adenyl cyclase activity in either liver membranes or in ghosts of fat cells which also contain a glucagon-sensitive enzyme system. The product, des-his-glucagon (DH-glucagon), inhibits both the action of glucagon on these systems and the binding of labeled glucagon to liver plasma membranes. The apparent affinity of DH-glucagon for the regulatory component is about 6-fold less than native glucagon indicating that histidine contributes to the forces involved in binding of the hormone.

Glucagon retains biological activity when two amino acids are removed from the COOH-terminus and methionine is substituted with homoserine at

position 27. However, cleavage of glucagon between residues 21 and 22 results in products that are not biologically active, even though NH_2-terminal histidine is present, and do not bind to the regulatory component. Fragments of glucagon encompassing residues 20-29 also do not bind to the regulatory component. Thus, nearly the entire glucagon molecule is essential for structure and function. The COOH-terminal region (20-29) is essential though not uniquely involved in binding of the hormone; the NH_2-terminal histidine is essential for biological action.

The COOH-terminal region is intensely hydrophobic and contains a tryptophan chromophore at position 25. Dr. Edelhoch and colleagues have found that the tryptophan chromophore of glucagon displays marked changes in fluorescence in the presence of detergents and have concluded that the detergents bind to hydrophobic regions surrounding the tryptophan residue and induce folding of the essentially structureless hormone. X-ray diffraction studies also indicate that the COOH-terminal hydrophobic region is a potential region of helical structure under appropriate environmental conditions.

Since the COOH-terminal region is essential for binding of glucagon to the regulatory component and can acquire structure in a hydrophobic environment, we have proposed that the COOH-terminal hydrophobic region of glucagon is the initial site of binding of the hormone. As indicated above, the regulatory component behaves as a lipoprotein and binds glucagon through forces easily disrupted by agents or conditions that alter hydrophobic interactions. It is further suggested that the initial hydrophobic binding induces conformational changes in the remainder of the glucagon molecule, possibly in a hierarchial fashion, that result in a thermodynamically stable complex between glucagon and the regulatory component. The expression of the formed complex is a unique biological activity, the physico-chemical basis of which is completely unknown, but which is ultimately reflected by the activation of adenyl cyclase.

Significance to Bio-medical Research and the Program of the Institute:
Adenyl cyclase is the key regulatory system for the initial actions of a number of peptide hormones and biogenic amines. It is, accordingly, fundamental for an understanding of the molecular basis of hormone action to have complete knowledge of the physico-chemical nature of this system. The studies outlined indicate that the glucagon-sensitive adenyl cyclase system is part of the plasma membranes of hepatic cells. It is a finely controlled system consisting of regulatory and catalytic components which interact in response to glucagon such that the message contained within the hormone is transferred and amplified by the system to give a new signal, 3'5' cyclic AMP, the so-called second messenger of hormone action. Of fundamental importance were the findings that activation of adenyl cyclase by glucagon is a function not only of the concentration of hormone exposed to the system but also of the concentration of nucleotides (GTP or ATP). The nucleotides serve the function of converting the regulatory component into an active state that binds glucagon only transiently thereby converting the enzyme system into rapidly reversible states of activity that depend upon continual supply of

hormone for maintenance of activity. Thus, the response of the liver cell to glucagon is not simply a function of hormone concentration but also of those cellular factors, including guanyl and adenyl nucleotides, that control the response. Future investigations must be concerned, therefore, with the levels of guanyl or adenyl nucleotides available to the system in the cell and those factors, hormonal or otherwise, that may influence their concentrations and thus their actions. Out of such studies may come answers to questions concerning resistance of a cell to hormone action, decay in response in spite of continued supply of stimulating concentrations of hormone, and an array of problems concerning how different hormones act either synergistically or in opposing fashion.

The structure-function relationships found in the glucagon molecule provide new clues into the physico-chemical basis for hormone action and of the inter- and intramolecular forces that determine structure and function in macromolecules. Future studies of structure-function relationships in glucagon and secretin, which have markedly similar primary structures should assist in unravelling the molecular basis for specificity of hormone action and of the nature of receptors that react with these hormones.

Proposed Course: Continued efforts will be made to isolate and characterize the various components of the glucagon-sensitive adenyl cyclase system. The role of nucleotides in the actions of other hormone-sensitive adenyl cyclase systems will also be investigated. Synthesis of analogues of glucagon and secretin, and chemical modifications of these hormones are being carried out and will be increasingly stressed. As progress is made on isolation of the enzyme system, it is hoped that more sophisticated physical methods will be available for studying interactions between hormones and the regulatory components of the enzyme system.

Honors and Awards: None

Publications:

Pohl, S.L., Birnbaumer, L., and Rodbell, M. Glucagon-Sensitive Adenyl Cyclase System in Plasma Membrane of Hepatic Parenchymal Cells. Science, 164, 566 (1970).

Birnbaumer, L., Pohl, S.L., Krans, H.M.J., and Rodbell, M. The Actions of Hormones on the Adenyl Cyclase System. Advances in Biochemical Psychopharmacology, Vol. III, 85 (1970).

Rodbell, M., Birnbaumer, L., Pohl, S.L., and Krans, H.M.J. Properties of the Adenyl Cyclase System in Liver and Fat Cells: The Mode of Action of Hormones. in: Luft and Randle, P.J. (Editors) Acta Diabetologica Latina. Vol. VII. Supplement 1, (1970) p. 9.

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Rodbell, M., Krans, H.M.J., Pohl, S.L., and Birnbaumer, L. The Glucagon-Sensitive Adenyl Cyclase System in Plasma Membranes of Rat Liver IV. Effects of Guanyl Nucleotides on Binding of ^{125}I-glucagon. J. Biol. Chem., 246, 1872 (1971).

Rodbell, M., Birnbaumer, L., Pohl, S.L., and Krans, H.M.J., The Glucagon-Sensitive Adenyl Cyclase System in Plasma Membranes of Rat Liver V. An Obligatory Role of Guanyl Nucleotides in Glucagon Action. J. Biol. Chem., 246, 1877 (1971).

Rodbell, M., Birnbaumer, L., Pohl, S.L., and Sundby, F. The Reaction of Glucagon with its Receptor: Evidence for Discrete Regions of Activity and Binding in the Glucagon Molecule. Proc. Nat'l Acad. Sci., 68 (1971).

Rodbell, M. The Role of Guanyl Nucleotides in Hormone Action at the Receptor. In: Walaas, O. (Editor) Symposium on Mechanism of Insulin Action. Scandanavian Society for Diabetes (1971) p. 1.

Serial No. NIAMD-LNE-7
1. Nutrition and Endocrinology
2. Nutritional Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemical Studies Related to Nutrition

Previous Serial Number: NIAMD-LNE-7

Principal Investigators: Drs. J. G. Bieri, J. N. Williams, Jr., and H. G. Windmueller

Other Investigators: Dr. I. R. Peake, S. L. Thorp and A. E. Spaeth

Cooperating Units: Dr. S. Eisenberg, MD, NHLI
Dr. Peter Herbert, MD, NHLI

Man Years

Total:	9
Professional:	7
Other:	2

Project Description:

Objectives - To determine the nutritional, biochemical and physiological role of essential nutrients for experimental animals. To define the metabolic function of certain nutrients and to study nutritional effects on the tissue levels of various metabolites.

Methods Employed - Rats, hamsters and guinea pigs are fed specially prepared, highly purified diets that contain adequate amounts of each nutrient known to be required by the particular species. The effects of specific deficiencies and imbalances are assessed by measurement of physiological, chemical and enzymological changes in the animal, its tissues and excreta.

The relationship of nutrition to other scientific areas, such as neurology and pathology, is studied.

Major Findings

A. Metabolism and Function of Fat-Soluble Vitamins. (J. G. Bieri and I. R. Peake)

Patients with a-betalipoproteinemia, who initially had zero plasma levels of vitamin E (α-tocopherol), were able to absorb sufficient amounts of large oral doses of the vitamin to permit low, but detectable, plasma concentrations. The red cells, however, had a normal content of α-tocopherol and

did not hemolyze in vitro. Because little is known of the relationships between plasma and other tissue concentrations of vitamin E, studies were initiated with rats. Young, rapidly growing rats were fed high doses of α-tocopherol for 13 days. The supplements were then stopped and groups of animals were killed at 1 to 2 week intervals for 45 days. A similar experiment was done with mature rats which had received a low dietary intake of the vitamin from birth. Analyses of tissues for α-tocopherol showed that during the first 8 to 15 days following removal of the dietary vitamin, the concentration fell markedly in the plasma, liver, heart and depot fat, the tissues with the highest initial concentrations (12 to 20 μg/g). After 15 days, the rate of loss was much slower. Muscle and testis had much lower initial concentrations (4 to 5 μg/g) and also depleted at a slower rate. The data indicate that liver, heart and fat have two pools of α-tocopherol, one very labile and one relatively fixed. It was also apparent that plasma concentrations do not accurately reflect tissue concentrations of the vitamin. (Dr. Bieri)

Studies were undertaken to compare the absorption and metabolism of α- and γ-tocopherols in an attempt to explain their different biological activities (5:1). In in vivo experiments, rats were injected intraperitoneally with mixtures of dl-[5-Me-^{3}H]-α-tocopherol and dl-[2,3-^{14}C]-γ-tocopherol. Tissue analyses at various times after injection indicated approximately equal uptakes of the tocopherols, but a more rapid metabolism of γ-tocopherol. In the liver and spleen there accumulated a dimer of γ-tocopherol but not of α-tocopherol. In in vitro experiments, the uptake by vitamin E deficient red blood cells of a 1:1 mixture of the two tocopherols in plasma was studied. There was a greater affinity by the red cell for γ-tocopherol than α-tocopherol. At equilibrium there was 36% uptake of γ-tocopherol and 28% of α-tocopherol. In similar experiments in which a synthetic tocopherol emulsion replaced plasma, a similar greater affinity of the red cell for γ-tocopherol was found. In collaborative studies with Dr. Windmueller, the intestinal absorption of α- and γ-tocopherol has been compared using rats with mesenteric lymph cannulae. After infusion of tocopherol in 15% fat emulsion directly into the stomach, 45% to 50% of the α-tocopherol was recovered in the lymph in 24 hours of collection. Gamma-tocopherol was consistantly absorbed at 85% the rate of α-tocopherol absorption. The degree of unsaturation of the infused fat had no significant effect on tocopherol absorption. (Dr. Peake)

B. Biochemical Studies of Cytochromes (J. N. Williams, Jr.)

We have continued studies of a sub-population of particles in purified liver mitochondria which bind cytochrome c more tenaciously than the bulk of subcellular particles. Using continuous sucrose gradient (isopycnic) methods of separation, it was found that the particles band very differently from the bulk of mitochondria and microsomes. Although the particles have strong cytochrome oxidase and monoamine oxidase activities (markers for mitochondria per se), they also possess enzyme activities characteristic of microsomes (G-6-Pase and NADPN-cytochrome c reductase). Their content of RNA is also relatively high. When rats were injected with phenobarbital, a reagent which

causes proliferation of the endoplasmic reticulum, the concentration of these particles increased. These particles may be immature mitochondria in which the outer membrane and endoplasmic reticulum are not yet separated.

Other studies are continuing on the mode of binding of cytochrome c to the outer and inner membranes of mitochondria. The outer membrane has approximately 6 times as many high affinity binding sites as does the inner membrane. Exogenous cytochrome c was found to penetrate the outer membrane and reach reactive sites on the inner membrane, an observation consistent with the theory that cytochrome c is made in the endoplasmic reticulum and then is incorporated into the mitochondria.

The effects of other substances high in lysine, as is cytochrome c, (L-lysine, low and high molecular weight poly-L-lysine, poly-L-ornithine, and bovine serum albumin) on the binding of cytochrome c to mitochondria were investigated. It was found that L-lysine had no effect on cytochrome c binding, low molecular weight poly-L-lysine was fairly effective in inhibiting binding, whereas high molecular weight poly-L-lysine and poly-L-ornithine prevented cytochrome c binding almost completely. Bovine serum albumin had an intermediate effect. High molecular weight poly-L-lysine was able to displace about one-half of the endogenous cytochrome c found in native mitochondria. Double reciprocal plots of exogenous cytochrome c bound to mitochondria *versus* added cytochrome c indicate that at low levels of high molecular weight poly-L-lysine the inhibition was competitive. When higher levels of inhibitor were added, the type of inhibition became noncompetitive. A similar inhibitory pattern by this compound was found for cytochrome c binding to microsomes. When effects of high molecular weight poly-L-lysine on the activities of mitochondrial enzyme systems requiring reduced or oxidized cytochrome c were studied, it was found that cytochrome oxidase, which utilizes reduced cytochrome c, was inhibited competitively by poly-L-lysine, whereas succinate-cytochrome c reductase was inhibited noncompetitively. In the latter case a plot of 1/v *versus* inhibitor concentration gave curves for different levels of substrate which did not differ significantly but which were also concave upward. Analysis of these curves indicated that the data fitted the equation $v/v_i = 1 + \frac{(I)^r}{K_i}$, in which r is 2, indicating that 2 molecules of inhibitor combine with one "molecule" of the enzyme system. The point or points where the inhibitor combines is under study at present.

C. Synthesis and Metabolism of Circulating Lipoproteins (H. G. Windmueller)

Studies are continuing to determine the sites of synthesis and metabolic fate of the various protein moieties of circulating lipoproteins, the transport vehicle for lipids throughout the body. Previous studies with intact animals have indicated that liver and intestine are responsible for most or all lipoprotein production, but the relative synthesis by these two organs of the specific lipoprotein apoproteins remains unknown. This question can now be approached with organ perfusion procedures developed recently in this laboratory for isolated rat liver and isolated rat intestine. The viability and

functional integrity of the latter preparation have been established from measurements of O_2 consumption, glucose and water transport, glucose utilization, lactate production, and lymph production. Furthermore, as a prerequisite to studying lipoprotein synthesis, it has been demonstrated that this isolated preparation can perform all the necessary steps in the complex fat transport process, including luminal fat digestion, absorption of digestion products, intracellular re-esterification and assembly of lipids and proteins into chylomicrons and smaller lipoproteins, and release of these products into lymphatic channels. This is the first such demonstration in vitro. During fat transport, isolated intestine incorporated radioactive amino acids into the proteins of chylomicrons and low density lipoproteins, which all appeared in lymph, and into the protein of high density lipoprotein, which appeared in equal amounts in lymph and blood. Lipoproteins accounted for nearly half of the newly-synthesized lymph protein. In collaboration with Dr. Peter Herbert (NHLI), we are proceeding with identification of the specific lipoprotein apoproteins synthesized by isolated intestine as well as by isolated liver.

In studies with Dr. S. Eisenberg (NHLI), the fate of circulating lipoproteins is being studied in rats injected intravenously with human plasma lipoprotein apoproteins labeled with I^{125}. Preliminary results indicate that the injected proteins become incorporated into rat plasma lipoproteins, and that liver is the major site of removal from the circulation.

D. Significance to Bio-Medical Research and the Program of the Institute

A more complete understanding of the nutrition, biochemistry and metabolism of essential amino acids, proteins, vitamins, minerals and fatty acids in different living organisms can be expected to contribute still further to our knowledge of the roles of these essential nutrients in human beings. It is well established that the nutrition of man plays a role in the etiology of many degenerative and metabolic diseases, certain infectious and neurological diseases and some types of cancer. Basic studies in nutrition and biochemistry of nutrients may provide the means to prevent or cure some of these diseases.

E. Proposed Course of Project

Clarification of nutritional responses and explanation of interrelationships at the biochemical level remain the primary goals of the project.

F. Publications

Ahluwalia, B. and Bieri, J. G.: Local stimulatory effect of vitamin A on spermatogenesis in the rat. J. Nutrition 101: 141-152, 1971.

Bersot, T. P., Brown, W. V., Levy, R. I., Windmueller, H. G., Fredrickson, D. S. and LeQuire, V. S.: Further characterization of the apolipoproteins of rat plasma lipoproteins. Biochemistry 9: 3427-3433, 1970.

Bieri, J. G.: Biological activity and metabolism of N-substituted tocopheramines: Implications on vitamin E function. In Deluca, H. F. and Suttie, J. W. (eds.): The Fat-Soluble Vitamins, Madison, Wisconsin, The University of Wisconsin Press, 1970, pp. 307-316.

Bieri, J. G. and Poukka, R. K. H.: Red cell content of vitamin E and fatty acids in normal subjects and patients with abnormal lipid metabolism. Internat. Z. Vit. Forschung. 40: 344-350, 1970.

Poukka, R. K. H. and Bieri, J. G.: Blood α-tocopherol: Erythrocyte and plasma relationships in vitro and in vivo. Lipids 5: 757-761, 1970.

Williams, J. N., Jr.: Response of the liver to prolonged protein depletion VII. Oxidative phosphorylation. J. Nutrition, in press.

Williams, J. N., Jr. and Thorp, S. L.: Influence of degradative procedures, salts, respiratory inhibitors, and gramicidin on the binding of cytochrome c by liver mitochondria. Arch. Biochem. Biophys. 141: 622-631, 1970.

Windmueller, H. G. and von Euler, L. H.: Prevention of orotic acid-induced fatty liver with allopurinol. Proc. Soc. Exp. Biol. Med. 136: 98-101, 1971.

Serial No. NIAMD-LNE-8
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Structure and function of barnase, an extracellular ribonuclease (barnase) from *Bacillus amyloliquefaciens*.

Previous Serial Number: Same

Principal Investigator: Dr. Robert W. Hartley

Other Investigators: None

Cooperating Units: Large-scale laboratory, Office of Chief, LNE, NIAMD
Dr. David R. Davies, LMB, NIAMD

Man Years

Total:	2.3
Professional:	1.0
Other:	1.3

Project Description:

Objectives: To further purify, characterize, and investigate this enzyme and its natural inhibitor by physical, chemical, and biological techniques. To develop methods of culture, genetic manipulation and genetic analysis so that the organism and its product enzyme-inhibitor pair may be applied to a broad study of enzyme structure, function, and synthesis.

Methods Employed: *B. amyloliquefaciens* (strain H and derivatives and several other strains all of which were formerly considered to be strains of *B. subtilis*) are grown in synthetic media in a variety of batch and continuous flow fermentations. Isolation of barnase involves: 1) adsorption onto P-cellulose from acidified culture medium, followed by stepwise elution, 2) desalting by Sephadex chromatography, 3) gradient chromatography on CM-cellulose. The inhibitor (barstar) is obtained from the cells after acetone and aqueous acid extractions by extraction at pH 8.5. It is purified by acid precipitation, DEAE-cellulose, and Sephadex G-75 chromatography and by making use of its specific binding to purified enzyme.

Work on the determination of amino acid sequence involves amino acid analysis, enzymatic digestions, peptides separations, and peptide sequencing by the combined Edman-dansyl techniques. Amino acid analysis is by automated liquid chromatography, using a JEOLCO analyzer. Dansyl amino acid derivatives are identified by thin layer chromatography on polyamide layers. Peptide separations are obtained by Sephadex and ion-exchange chromatography.

The reversible transition undergone by barnase and its derivatives under the influence of heat or denaturing agents is studied primarily by ultraviolet absorption, but also by optical rotatory dispersion, exclusion chromatography, and with the use of proteolytic enzymes.

Derivatives, such as those prepared by partial exopeptidase digestion, are also compared to native barnase with respect to activity, reaction with inhibitor and with anti-enzyme antibody, chromatographic behavior, crystal growth, etc.

Equilibrium ultracentrifugation has been used to measure the molecular weights of the enzyme, the inhibitor, and their complex, and to determine the molecular weight homogeneity of each.

Disc and equilibrium pH gradient electrophoresis have also been used to assess homogeneity and to demonstrate the reversibility of the complex formation.

Bacterial mutants are obtained by chemical mutagenesis of vegetative cells. and by gamma irradiation of spores. A variety of techniques are used to isolate ribonuclease, protease, amylase, nutritional, and antibiotic resistant mutants.

Major Findings: The amino acid sequence of barnase is as shown.

Ala-Gln-Val-Ile-Asn-Thr-Phe-Asp-Gly-Val-Ala-Asp-Tyr-Leu-Gln-Thr-Tyr-His-Lys-Leu-Pro-Asn-Asp-Tyr-Ile-Thr-Lys-Ser-Glu-Ala-Gln-Ala-Leu-Gly-Trp-Val-Ala-Ser-Lys-Gly-Asn-Leu-Ala-Asp-Val-Ala-Pro-Gly-Lys-Ser-Ile-Gly-Gly-Asp-Ile-Phe-Ser-Asn-Arg-Glu-Gly-Lys-Leu-Pro-Gly-Lys-Ser-Gly-Arg-Thr-Trp-Arg-Glu-Ala-Asp-Ile-Asn-Tyr-Thr-Ser-Gly-Phe-Arg-Asn-Ser-Asp-Arg-Ile-Leu-Tyr-Ser-Ser-Asp-Trp-Leu-Ile-Tyr-Lys-Thr-Thr-Asp-His-Tyr-Gln-Thr-Phe-Thr-Lys-Ile-Arg.

Barnase has been covalently bound to agarose (using the cyanogen bromide procedure) in such a manner that ∿65% of molecules are active in binding the inhibitor, barstar. This has made affinity chromatography a practical procedure in the purification of barstar, and some 15-20 mg have indeed been purified with its aid.

Rabbit antisera have been prepared against both barnase and barstar, providing a useful tool for studying the complex and for investigating mutant forms of both proteins.

Significance to Bio-Medical Research and the Program of the Institute: The small size, stability, easily measured enzyme activity and absence of disulfide bridges in barnase made it an excellent choice for a long-range study of the relation between the structure of an enzyme and its function. It now appears that the inhibitor is an even simpler protein and should be useful in a similar fashion. Together they offer a model system for the study of a protein-protein interaction.

The nature and extent of the thermal transition of barnase gives strong

support to the concept that the three dimensional structure of a protein may be completely determined by its amino acid sequence. The simplicity and all-or-none nature of the transition should make it useful for thermodynamic analysis of the factors involved in maintaining a folded protein structure.

The production of both of these proteins by the same organism is also of biological interest.

Proposed Course of Project: Further characterization and study of the three entities - enzyme, inhibitor and complex - and various derivatives of each will be carried out by as many techniques as possible. Determination of the amino acid sequence of barstar is a must. Attempts to prepare crystals suitable for x-ray work will continue.

Comparison studies by the same techniques will also be applied to the homologous proteins produced by other independently isolated strains of *B. amyloliquefaciens*. It is a hope that similar studies can be applied to altered proteins produced by genetic variants of these strains.

Honors and Awards: None

Publications:

Hartley, R. W.: Derivatives of *B. amyloliquefaciens* Ribonuclease (barnase) isolated after limited digestion by carboxypeptidases A and B. Biochem. Biophys. Res. Comm. 40, 263-270, 1970.

Serial No. NIAMD-LNE-9
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on structure and function of nucleic acids.

Previous Serial Number: Same

Principal Investigator: Dr. G. W. Rushizky

Other Investigators: Dr. H. A. Sober, Mr. J. E. Mozejko
Mr. D. L. Rogerson, Jr.

Man Years

Total:	2.3
Professional:	2.3
Other:	0

Project Description:

Objectives: To study the enzymology and chemistry of nucleic acids and to develop new methods for the determination of nucleotide sequences in RNA and DNA.

Methods Employed: Various systems for paper electrophoresis, paper chromatography, and polyacrylamide gel electrophoresis were examined with a view toward developing a fractionation procedure for tRNAs as well as similar oligonucleotides derived from high molecular weight nucleic acids.

Other experiments dealt with the column chromatography of partial and complete enzymatic digests of RNA and DNA. The adsorbents DEAE-Sephadex and DEAE-cellulose were used with buffers containing either 7 M urea or 50% MeOH at neutral or acid pH. A useful sequence of these systems consisted of a) fractionation in 7 M urea at neutral pH according to chain length, b) subfractionation of compounds of equal chain length according to Gp content, in 50% MeOH at pH 5.2, and c) further fractionation according to base ratios of compounds of equal chain length and Gp content in 7 M urea at pH 3.2.

Major Findings: Two-dimensional polyacrylamide gel electrophoresis was found to be superior to electrophoresis and/or chromatography with filter paper or ion-exchange paper for tRNA resolution. (Better than 85% pure arginine- and F-methylmethionine tRNA were obtained from Dr. A. D. Kelmers, Oak Ridge National Laboratories). The best separations of the two tRNAs charged with the proper amino acid, were obtained at pH 3.2 - 4.2 with a modification of the acid pH gel catalyst system of Raymond, in 8-12% gels and 3-6 M urea. Separations of both tRNAs (uncharged) were also obtained in the presence of 0.01 M Mg, or when the tRNAs were complexed with Hg^{2+}. This system will be extended to

mixtures of all tRNAs labeled with ^{14}C amino acids. Counting of gels fractions should be facilitated by the arrival of a new scintillation counter.

Column chromatography of oligonucleotides on DEAE-Sephadex in 7 M urea affords separations according to chainlength only. The use of 50% MeOH at pH 5.2 permits further fractionation of oligonucleotides up to the pentamer level not only by chainlength, but also on the basis of Gp content. This is useful for the fractionation of digests of RNA with enzymes that yield compounds of equal chainlength, but differing Gp content, such as with the RNase of Ustilago sphaerogena, of B. amyloliquefaciens, or of Micrococcus aureus. The latter enzyme is especially useful for RNA digests since it alone does not produce 2',3'-cyclic terminal phosphate intermediates. Pure 3'-terminal phosphate forms of ACC, UCC, UUC, AUC,(CGC), (UGA), (UGC), CGG, AGG, and UGG were thus isolated from micrococcal nuclease digests of RNA.

The Ustilago and B. amyloliquefaciens enzymes also produce several of these trimers, but in various proportions of 2',3'-cyclic terminal - as well as 3'-phosphate forms. Acid hydrolysis does not completely open the phosphate triesters, and produces mixtures of 2'- and 3'-terminal phosphate forms. The pH 5.2 - 50% MeOH-DEAE-Sephadex system does not distinguish between 2',3'-terminal phosphate and 2'- or 3'-terminal phosphate forms of trinucleotides.

The fractionation of oligonucleotides on DEAE-Sephadex in 50% MeOH is also of interest with enzymatic digests of DNA since no specific nucleases for DNA are known. Enzymes such as micrococcal nuclease have a preference, but no strict specificity, for linkages adjacent to Ap and Tp. The method sequence a) - c) (see methods) yielded, among the DNA-trimers, preparative amounts of such compounds as (ACC), (AAC), TCC, AAA, (ACT), TTC, AAG, (AGC),(CGC), (TGA), (TGC), TTG, CGG, AGG, and TGG.

Significance to Bio-Medical Research and the Program of the Institute: An additional mapping procedure for the fractionation of large oligomers such as tRNA should be useful for characterization of tRNAs, as well as for nucleotide sequence studies of high molecular weight RNA or DNA.

Methods for the extensive column chromatography of small oligomers are of interest for the preparation of pure trimers as model compounds, as well as for the characterization of preparative amounts of partial enzymatic digests of RNA and especially of DNA.

Honors and Awards: None

Publications:

Rushizky, G. W., Mozejko, J. H., Roberson, D. L. Jr., and Sober, H. A.: Characterization of enzymatic specificity of a RNase from Ustilago sphaerogena. Biochemistry 9: 4966-4970, 1970.

Rushizky, G. W., and Mozejko, J. H.: Fractionation by column chromatography in aqueous methanol of partial Ustilago sphaerogena nuclease digests of RNA. Anal. Biochem. In press, 1971.

Serial No. NIAMD-LNE-10
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Protein:Nucleic acid interaction: Chromatin structure and restriction in eukaryotic cells.

Previous Serial Number: Same

Principal Investigator: Dr. Robert T. Simpson

Other Investigators: Dr. Herbert A. Sober

Cooperating Units: None

Man Years

Total:	1.0
Professional:	1.0
Other:	0

Project Description:

Objectives: To study the structural and enzymatic bases for the restriction of the translation of the major portion of the genetic information in nucleated, mammalian cells; and to study the mechanisms which allow translation of that portion of the genetic message which is appropriate to the function of the particular cell type.

Methods Employed: Using established methodology, nuclei are isolated from mammalian cells, and then chromatin, the diffuse interphase form of the chromosomes, is isolated from these nuclei. The physicochemical properties of the isolated chromatin are studied using standard biochemical techniques, such as selective chemical modification, circular dichroism, absorption spectrophotometry, ultracentrifugation, hydrogen ion titration, and interaction with reporter compounds.

Major Findings: During the past year, primary effort has been directed to the understanding of the topographical interrelationships of DNA and proteins in chromatin. Investigation of the geometry and stoichiometry of the binding of a reporter compound $2,4\text{-}(NO_2)_2C_6H_5NH(CH_2)_2N^+(CH_3)_2(CH_2)_3N^+(CH_3)_2\cdot 2Br^-$, to native DNA and to chromatin have indicated qualitatively similar and quantitatively identical binding to the free and complexed nucleic acids. From the work of others, this molecule is known to bind in the minor groove of the DNA double helix, and thus these results demonstrate that the proteins of the chromatin complex must bind only in the major groove. The minor groove is thus left bare, potentially capable of interacting with other nucleic acids, or

enzymes which are able to recognize nucleotide sequences.

In order to study the details of binding of the histones to DNA in chromatin, the nucleoprotein complex has been acetylated with acetic anhydride. Under appropriate conditions, a limited number of lysyl residues of the histones are modified by this reagent - these are presumably the lysyl groups not involved in binding of the proteins to DNA. Native and acetylated chromatin are essentially identical when examined by various physicochemical techniques, and none of the proteins of the nucleoprotein complex are dissociated consequent to the modification. Gel electrophoresis demonstrates that all the histones are acetylated partially, and suggests that the modification reaches only a certain select group of lysyl residues of each individual histone molecule.

Studies to localize the modified residues in the primary sequences of the various histones are currently in progress. Early results for the slightly-lysine-rich histone, FII, indicate that the nonbound lysyl residues are localized in a long sequence in the middle portion of the molecule, a sequence which contains relatively few basic residues, and most of the hydrophobic amino acids of this protein. These findings suggest that the carboxyl and amino terminal portions of the histone are bound to DNA, while the central region either loops out or forms a bridge between two portions of a single DNA chain or two separate DNA chains. If bridges are formed, it is of particular interest, since this histone class has been shown by our earlier work to be of prime import in the stabilization of the altered structure of DNA when it is present in chromatin.

Selective proteolysis of native chromatin has been employed in an attempt to ascertain whether the nonbound regions of the histones are merely looped out, or whether they form bridges of import in formation of the superstructure of the complex. Digestion of chromatin with trypsin cleaves polypeptide bonds in a number equal to the number of lysyl and arginyl residues titratable as "free" (or nonbound) in the nucleoprotein. Thirty percent of the protein of chromatin, including peptides derived from all the histones, is dissociated from DNA by such a digestion. The resultant chromatin is nearly identical to the native molecule in terms of thermal denaturation properties, but is increased in its intrinsic viscosity and flow dichroism, and has a circular dichroism pattern like that of DNA, in contrast to native chromatin. Thus, the properties of DNA in chromatin which have been attributed to local charge neutralization are not altered by tryptic removal of the nonbound portions of the histones and nonhistones; while those properties which have been equated with formation of a supercoiled structure for the DNA, are altered in the direction of the protein-free nucleic acid by such digestion. These results strongly suggest a bridging role for some of the histones in maintainance of the native conformation of chromatin.

Significance to Bio-Medical Research and the Program of the Institute: Our studies of the structure of chromatin, begun last year, are progressing to a point where we feel that it will soon be possible to construct meaningful models of the nucleoprotein complex. With this information in hand, reasonable questions concerning the alterations in this structure which occur consequent

to gene activation in eukaryotic systems, and alterations which occur concomitant with the dedifferentiation of a cell, will be able to be posed.

Honors and Awards: None

Publications:

Simpson, R. T. and Sober, H. A.: Circular dichroism of calf liver nucleohistone. Biochemistry 9, 3103-3109,1970.

Simpson, R. T.: Interaction of a reporter molecule with chromatin. Evidence suggesting that the proteins of chromatin do not occupy the minor groove of deoxyribonucleic acid. Biochemistry 9: 4814-4819, 1970.

Serial No. NIAMD-LNE-11
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Oligonucleotides: Properties and biological activity.

Previous Serial Number: Same

Principal Investigator: Dr. Pamela Woodford

Other Investigators: Dr. G. W. Rushizky and Dr. H. A. Sober

Cooperating Units: Dr. M. Sela, Department of Chemical Pharmacology, Weizmann Institute of Science, Rehovot, Israel
Dr. A. Rich, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts.

Man Years

Total:	1.3
Professional:	1.3
Other:	0

Project Description:

Objectives: To study the chemical, physical and biological properties of pure ribo-oligonucleotides of known sequence and chainlength.

Methods Employed: Yeast RNA nucleotides have been partially and completely digested by specific nucleases and the oligonucleotide products resolved and isolated by DEAE-cellulose and Sephadex chromatography at various pH values in urea and aqueous methanol buffers to achieve separation on the basis of chainlength and base composition. Purification has been achieved by rechromatography, and desalting. The isolated products have been examined by paper chromatography, paper electrophoresis, and spectral absorption techniques. Rabbits and guinea pigs have been immunized with some fo these trinucleotides combined with inert polyamino acids and the antibodies produced are being tested for nucleotide and sequence specificity.

Major Findings: Ribonucleotide trimers terminating in 3'-phosphates have been prepared from yeast RNA by *Ustilago* RNAse digestion using chromatographic techniques. The enzyme produces preferential cleavage of Ap-Py linkages. Trinucleotides in particular are important because they serve as codon and anticodon units for amino acid incorporation into proteins.

The following trinucleotides have been obtained: ACU, AUC, (UC)A, UUA, (UA)U, (CU)G, UUG, (UG)U, UGG, and CGG which, with trinucleotides already in hand, constitute complimentary triplet pairs. Of particular interest are

the last two trinucleotides since they can be used for the formation of complementary pairs.

Isolation of trinucleotide sequences is being done by *Ustilago* ribonuclease and aqueous methanol column chromatography. A large scale purification of ribonuclease T_1 is in progress.

Significance to Bio-Medical Research and the Program of the Institute: Large scale purification of ribonuclease T_1 to be used for the preparation of oligonucleotide fragments.

Large amounts of oligonucleotides are needed for the following studies planned or in progress.

1) Study of the physical and chemical properties and crystallization and x-ray diffraction studies on the individual trinucleotide and complementary pairs of trinucleotides so that background information may be obtained for the work on large oligonuclotide chains.

2) As reagents for the preparation of affinity adsorbents which can be used for such studies as localizing sequence specific nucleases.

3) Possible antigens for the production of sequence-specific antibodies.

4) As substrates for kinetic studies of nucelase action.

5) Analysis of circular dichroism of ribonucleotide trimers.

Large scale production of mammalian cell cultures and cell lines which can be used as sources of enzymes, nuclear material, and can be used for studies on enzyme action, hormone production, etc.

Honors and Awards: Hillebrand Award, 1970, Herbert Sober.

Publications:

Woodford, P. C., Roane, P. P., Ward, T. G., and Hansen, P. Arne: Characterization of a virus isolated from a case of human infectious hepatitis. Journ. Microbiol. In press, 1971.

Serial No. NIAMD-LNE-12
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Fractionation of chromatin and the study of nonhistone proteins.

Previous Serial Number: None

Principal Investigator: Dr. S. Levy

Other Investigators: Dr. R. T. Simpson
Dr. H. A. Sober

Man Years

Total:	1.3
Professional:	1.3
Other:	0

Project Description:

Objectives: Development of a general method for fractionation of chromatin, and the isolation and characterization of its acidic proteins. To study the possible role of nonhistone proteins in restriction of template activity of chromatin.

Methods Employed: Chromatin is isolated from cell nuclei by established methods, and fractionated by ultracentrifugation and ion exchange chromatography. The fractions obtained are analyzed using methods such as spectrophotometry, electrophoresis, amino acid composition and immunochemical assays.

Major Findings: A general method for the fractionation of chromatin was developed which avoids the use of acids and ionic detergents in the fractionation procedure. Chromatin components are separated according to both their molecular weight and their ionic character. Chromatin is dissociated using salt-urea and DNA spun down by ultracentrifugation, dissociating and separating 90% of the proteins from DNA. These proteins are further fractionated by ion exchange chromatography. Cation exchange chromatography resolves the distinct histone fractions; while the unretained acidic proteins and RNA are further fractionated on an anion exchanger, using salt gradient elution in the presence of urea. Further studies on these acidic proteins are now in progress.

There have been a number of reports of changes in the nonhistone components of chromatin which accompany or precede changes in protein synthesis. Thus far, none of these proteins has been isolated and characterized. The method developed enables extraction and fractionation of all chromatin components and the isolation of any particular fraction of interest, and hence the use of this fractionation method for the isolation of such proteins which may play

a role in genetic restriction in eukaryotic organisms will be attempted.

Significance to Bio-Medical Research and the Program of the Institute:

There is some evidence that specific restriction of chromatin template activity might be due to the nonhistone nucleoprotein components. The present study attempts to isolate and characterize these nucleoproteins and to further investigate their role in regulation of gene expression. Knowledge of the basis for restriction of gene expression is a prerequisite to the understanding of cellular development, differentiation, and dedifferentiation.

Honors and Awards: NONE

Publications:

Yaron, A., Fasman, G. D., Sober, H. A. and Berger, A.: The Chainlength Dependence of the Conformation for Oligomers of L-lysine in Aqueous Solutions: Optical Rotatory Dispersion Studies. Biopolymers, in press, 1971.

ANNUAL REPORT SUMMARY

LABORATORY OF BIOCHEMISTRY AND METABOLISM

Carbohydrate Metabolism

A. Mucopolysaccharidoses. The abnormal mucopolysaccharide metabolism of fibroblasts from patients with the genetic mucopolysaccharidoses is due to the absence of a specific protein. When this protein is supplied exogenously, the metabolism is restored to normal. To date, five such proteins (designated "factors") have been observed: one deficient in the Hurler syndrome, one in the Hunter syndrome, two in the Sanfilippo syndrome (only one, however, in any one Sanfilippo patient), and one in the Maroteaux--Lamy syndrome. These factors are present not only in fibroblasts and fibroblast secretions, but also in normal human urine. In addition, fibroblasts from patients with an atypical mucopolysaccharidosis, which have no detectable β-glucuronidase activity, have an abnormal mucopolysaccharide metabolism correctible by β-Glucuronidase can therefore be considered a sixth factor.

A thousand-fold purified Hurler factor has been shown devoid of activity toward all the other mucopolysaccharidoses. It is also devoid of most of the lysosomal enzymes which have high activity in urine, but is still contaminated with β-N-acetyl hexosaminidase and acid phosphatase. It is estimated that at least another ten-fold purification would be required to obtain the factor in pure form, implying that it is but a trace constituent of urine. [Dr. E. F. Neufeld].

B. Synthesis of Inositol. Because of its speed and sensitivity gas chromatography is an important analytical tool in this investigation. Although trimethylsilylation has been the method used for derivatization of sugars and cyclitols under study, its limitations have forced a search for better derivatives. A new reagent, n-butaneboronic acid, has been found which is superior in several aspects. In an extension of the application of this reagent to carbohydrates, in general, it was found that the three biologically important hexoses, glucose, mannose, and galactose can be separated as alditol n-butaneboronates in less than 1/10 the time required for the alditol acetates; the silyl ethers of those same alditols cannot be separated. [Dr. F. Eisenberg].

C. Chitin Synthetase. Chitin is concentrated, as a disk-like structure, in the yeast bud scar, i.e., at the site in the cell wall where the bud has detached from the mother cell. The chitin disk is sandwiched between layers of glucan, a major component of the cell wall. Thus, chitin may be considered as the specific component of the yeast septum. With synchronously growing cells, the synthesis of chitin takes place during only part of the cell cycle, namely between bud initiation and completion of the septum. Thus, the formation of chitin is initiated at a specific point in space and time. Newly discovered features of the enzymatic system concerned with chitin synthesis suggest how the triggering mechanism may operate.

When particulate preparations from Saccharomyces cerevisiae S2880 were submitted to mild sonic oscillation, the chitin synthetase activity was reduced to a very low value. Nevertheless, incubation of these particles with either the supernatant fluid from the sonic treatment, or with trypsin led to a 15 to 20-fold increase in activity. The activating factor present in the sonic supernate can be precipitated with ammonium sulfate and dialyzed. It is heat-labile and has therefore the characteristics of an enzyme. The heat-stable protein previously described as an inhibitor of chitin synthetase, has now been found to act as an inhibitor of the activating factor, which contaminated preparations of the synthetase. Kinetic evidence indicates a very tight binding between the inhibitor and the activating factor.

A working hypothesis has been formulated, to explain how the three components of the chitin system, i.e., the synthetase, the activating factor and the inhibitor, may interact in the formation of the cell septum. The results indicate that chitin synthetase is present in the cell mainly in an inactive state, the "zymogen" state. It is suggested that the zymogen is distributed more or less uniformly on the cytoplasmic membrane. At the time of septum initiation, vesicles carrying the activating factor would be directed at the bud site. Thus the factor would transform zymogen into active enzyme at the desired location only. The function of the inhibitor is less clear, but its presence in the soluble fraction suggests that it could serve to trap any activating factor that might be released in the cytoplasm, thus preventing chitin synthetase activation at any other than the prescribed site. [Dr. E. Cabib].

D. Glycoproteins. Evidence has been obtained to extend the earlier observation that removal of terminal sialic acid residues from ceruloplasmin resulted in the rapid uptake of this protein by the liver. Upon injection into rats all of the desialylated proteins tested, with the exception of transferrin, were promptly removed from the circulation and were recovered in the liver. the materials examined include orosomucoid (α_1 acid glycoprotein), fetuin, haptoglobin, α_2-macroglobulin, lactoferrin, thyroglobulin, human chorionic gonadotropin and follicle-stimulating hormone. Competitive inhibition of hepatic uptake was demonstrated by the injection of tracer amounts of ^{64}Cu-ceruloplasmin together with substantive amounts of the above-mentioned desialylated proteins or the glycopeptides derived from them. Uptake was not inhibited by the fully sialylated protein or their glycopeptides. Employing the competitive inhibition technique, additional support was provided for the identification of the hepatocyte, and not the Kupffer cell, as the site of active accumulation of asialo-glycoproteins.

A general method has been described for the radioactive labeling of proteins containing terminal sialic acid in the carbohydrate moieties. Quantitative conversion of these residues to a radioactive 7-carbon analogue of sialic acid was activated by sequential periodate oxidation and borohydride reduction. The resulting radioactive preparations of several naturally occurring glycoproteins exhibited a normal half-life in plasma and proved suitable for metabolic studies. Application of this technique to human chorionic gonadotropin and follicle-stimulating hormone resulted in retention of biological activity.

The major locus of binding for desialylated glycoproteins has been identified as the hepatic plasma membranes and the binding process has been shown to involve a dual role for sialic acid. The presence of the glycoprotein is incompatable with binding. Complete reversibility was accomplished by alteration of the calcium ion concentration and pH of the medium, thereby suggesting a possible model system for the binding and transport of proteins across the liver membranes. [Dr. G. Ashwell].

E. Immunoglobulins. Murine myeloma cells (ADJ-PC5), incubated in vitro with ^{3}H-leucine, secrete ^{3}H-immunoglobulin G (IgG) as a single molecular species as judged by the migration characteristics of the labeled product on sodium dodecyl sulfate-acrylamide gel electrophoresis. However, the fact that some of the interchain disulfide linkages of intracellular immunoglobulins had not been acquired permitted the identification of the following intracellular species: LHHL (identical to IgG), HHL, HH and L (H and L refer to heavy and light polypeptide chains, respectively). Although HH and HHL were readily observed, radioactivity was not detected in the region of the gel where HL would be expected. The smooth microsome fraction contained principally LHHL, while HHL and HH were the predominant components in the rough microsome fraction. Only LHHL was labeled when cells were incubated with ^{3}H-galactose and such immunoglobulins were found mainly in the smooth fraction. On the other hand, immunoglobulins labeled with ^{3}H-mannose and ^{3}H-glucosamine were confined principally to the rough microsomes and the label appeared not only in LHHL but in HH and HHl as well. These findings indicate that some sugar residues are acquired before the synthesis of certain of the disulfide bonds. Moreover, galactose appears to be acquired at a site distinguished from the intracellular site where some glucosamine and mannose residues are added to the partially synthesized immunoglobulin molecule. [Dr. M. Kern].

Ribonucleic Acid: Polynucleotide Phosphorylase

I. Structural Studies. The single most important accomplishment this year has been the introduction of chromatography on phosphocellulose for purification of polynucleotide phosphorylase. This has decreased to a large extent the time required to obtain pure enzyme and in addition, provides, for the first time, sufficient quantities of material to undertake detailed experiments in protein chemistry.

Additional evidence supporting the notion that the heterogeneity of pure, primer-independent polynucleotide phosphorylase (Form I) from Micrococcus luteus is the result of proteolysis during isolation has been obtained. The distribution and quantities of the various bands seen on disk electrophoresis varies from preparation to preparation. Nevertheless, the enzyme appears homogeneous in the ultracentrifuge suggesting that the various bands are the same size. Under denaturing conditions, anywhere from 2 to 4 subunits are detected, depending on the preparation. Different bands of undenatured enzyme, isolated from disk electrophoresis gels, have a different distribution, both quantitatively and qualitatively, of the subunits. The single band of enzyme produced by limited tryptic hydrolysis (Form T) of the isolated enzyme has only one subunit. Thus the structures of the original subunits are closely related and may indeed have been a single subunit prior to proteolysis during isolation.

Amino acid analysis of Form I, as well as titration of cysteine with ^{14}C-N-ethylmaleimide in reduced, denatured enzyme indicates that Form I has approximately ten cysteine residues per molecule. Reduction of Form I followed by treatment with N-ethylmaleimide under mild denaturing conditions results in the addition of three moles of N-ethylmaleimide per mole. Concomitant with this the enzyme becomes primer-dependent.

II. Mechanistic Studies. Extensive experiments using dADP and adenosine methylene diphosphonate as (AOPCP) inhibitors of the polymerization of ADP have yielded new insights into the mechanism of polynucleotide phosphorylase action. dADP is a powerful inhibitor of ADP polymerization at concentrations much lower than those of the ADP itself. With Form I it is a competitive inhibitor with a K_i of about 7 μM. Under the same conditions, AOPCP does not inhibit ADP polymerization at all. This is in marked contrast to the very similar effects of both dADP and AOPCP on oligonucleotide phosphorolysis. In the latter case both are competitive inhibitors with respect to P_i, with identical K_i values, namely 100 μM. Thus it appears that inhibition of polymerization by dADP involves a site on the enzyme other than that involved in oligomer phosphorolysis. It was proposed that in polymerization, the very sensitive inhibition is due to interference with initiation of de novo synthesis while the site with the higher K_i, which is sensitive to both dADP and AOPCP is the site involved in addition of monomers to the already growing chain. This interpretation is supported by the following additional findings. 1) With Form I, dADP specifically inhibits de novo polymer formation. 2) Addition of oligonucleotides overcomes dADP inhibition to a large extent and the reaction occurring is almost exclusively addition to oligomers. 3) Form T, which catalyzes the addition to oligomers more readily than does Form I is only very slightly sensitive to dADP inhibition in the presence of oligomer.

Albeit a strong inhibitor, dADP is also a substrate for polynucleotide phosphorylase. The enzyme catalyzes the exchange between the β-phosphate of dADP and inorganic ^{32}P. The exchange requires either oligomer or ADP and thus appears to proceed through intermediary steps of polymerization and phosphorolysis. Additional evidence against the alternative mechanism for exchange, namely formation of dAMP-enzyme complex, is derived from extensive but negative experiments attempting to detect such a complex.

That dADP can indeed take part in polymerization was demonstrated directly. A single dAMP residue is readily added to a preformed oligoribonucleotide primer. Further addition of dAMP or AMP is very difficult, indicating that the dAMP residue is a poor acceptor in the chain extension reaction. dAMP is also incorporated into polymer during de novo synthesis using a mixed substrate, of dADP and ADP. In this case, however, dAMP residues occur at internal positions in the resulting copolymer. The polymers are of high molecular weight. [Dr. M. Singer].

Sulfur Nucleotides: Biosynthesis. Of the two enzymes involved in the formation of 4-thiouridine in tRNA, the first enzyme has been purified a thousand-fold, the sulfur-transferase itself, one-hundred-fold. Neither is yet homogeneous, but no other thionucleotides are formed beside 4-thiouridine. The only acceptor thus far found is tRNA. There appears to be no specific base sequence requirement, and no acceptor activity is found

with synthetic polyribonucleotides (A, U, GU) or with multistranded complexes (A + U, A + U + U). Native or denatured DNA are likewise inactive. This specificity is probably a property of the first enzyme in the system, which "activates" the tRNA in preparation for the actual introduction of sulfur.

The second enzyme, the sulfur-transferase, contains pyridoxal phosphate. Inactivation by hydroxylamine can be reversed by pyridoxal phosphate, but not by pyridoxal, pyridoxamine or its phosphate. Inactivation also results from KBH_4 treatment (which reduces the PLP aldehyde or its Schiff base) or from incubation with amino acids (presumably through transamination to form the inactive PMP-enzyme). The apo- and holo-enzymes may be resolved on hydroxylapatite. [Dr. M. Lipsett].

Hormone Dependent Differentiation of Mammary Gland. Insulin plays several key roles in the development of the mouse mammary gland, in vitro. It promotes epithelial cell proliferation, a process which appears to be necessary for differentiation. It plays an essential part, together with corticosteroid, in the formation of rough endoplasmic reticulum (RER). It also is required in order for prolactin to exert its functions on cells which had previously been exposed to both insulin and corticosteroid. Yet, in the intact virgin animal neither endogenous insulin nor corticosteroid exert these effects on the mammary epithelial cells. This, it turns out, is because the mammary cells in the non-pregnant animal are not sensitive to insulin. Insulin-sensitivity develops by the second day of pregnancy. Furthermore, it appears that the elevation in the levels of corticosteroids which occurs shortly after the start of pregnancy is at least partly responsible for conferring insulin-sensitivity on the epithelial cells. This effect of corticosteroids has been demonstrated both in vivo and in vitro. Thus, corticosteroids make it possible for insulin to act, and insulin, in turn, makes it possible for the corticosteroids to participate in the formation of the RER. [Dr. Y. Topper].

Enzymatic Utilization of Model Compounds. These studies have continued a two-fold emphasis on the metabolic utilization of a variety of model compounds bearing specific functional groups , as well as on those features of proteins responsible for specific catalysis.

In the former case are studies with epoxides which have resulted in the isolation of enzymes specific for the cleavage of such groups. An enzyme from mammalian liver catalyzes the ring cleavage of both aliphatic and aromatic epoxides in the presence of glutathione and yields the thioether of glutathione and the epoxide. The thioether, in turn, is expected to be cleaved of its glycine and glutamic acid, two of the three amino acids which constitute glutathione, and then to be acylated at the site of the third amino acid, cysteine. The result is a group of compounds generically termed, mercapturic acids. Work with the glutathione-linked epoxidase has resulted in an enzyme preparation which is essentially homogenous. The purified preparation is being used in the determination of the physical and catalytic parameters of the enzyme.

Contributions in the latter groups involve an extensive study of yeast aldehyde dehydrogenase, an enzyme composed of four subunits more than one of which is active. The catalytic reaction, i.e., conversion of aldehyde to acid, occurs after attachment to the protein of first aldehyde and then pyridine nucleotide. Inhibition of enzyme by one of the products of the reaction, reduced pyridine nucleotide, is being used as a means of approaching other aspects of the catalytic mechanism. One group of studies implicates a role for two sulfhydryl groups which are in close juxtaposition to each other. [Dr. W.B. Jakoby].

Enzyme Indication. A Lac Permease--An overproduction of lac permease is induced in E. coli carrying a defective lac transducing phage. Derivatized polyacrylamide and polysaccharide adsorbants containing a specific thiogalactoside having a high affinity for the lac permease are used to selectively extract and purify the lac permease carrier protein.

The heptaacetyl derivative, methyl (2,3,4,6 tetra-O-acetyl β-galactopyranosyl 2,3,4, tri-O-acetyl-1-thio β-galactopyranoside) uronate has been synthesized and characterized by elementary, O-acetyl and NMR analysis. Deacetylation has yielded a crystalline ester, not yet characterized, which has a high affinity for the lac permease.

B. Synthesis of 4-methyl-β-hydroxymethyl thiazole. Chemical mutagens are used to generate cells resistant to high concentrations of the thiamine antagonist "2-amino thiazole." Resistant strains producing an excess of thiamine thiazole are detected in cross-feeding experiments with thiazole requiring mutants of the same test organism.

Approximately 20 mutants resistant to 0.01 M "2-aminothiazole" have been isolated. Several of these appear capable of cross feeding a thiazole requiring test strain. [Dr. I. Leder].

Reductive Metabolism of cysteine in cystinotic cells. Whole cell extracts of normal human leukocytes are able to catalyze enzymatically the reduction of cystine, as well as other disulfide-containing substances, in the presence of reduced glutathione. The predominant proportion of this activity was found to be present in the soluble (cytoplasmic) fraction of the cell, with little or no activity detectable in the granular fractions. No difference in enzyme content as between normal and cystinotic cells was apparent in these studies. [Dr. F. Tietze].

Thermodynamic and Kinetic Studies of Enzymes. Kinetic experiments on fact reactions notably the dye/DNA reaction are carried out in a combined "T-jump-Stop-flow" device obtained from the American Instrument Company. Slower reactions such as the thermal unfolding of ribonuclease, which takes place over several seconds are being studied in a "slow-T-jump" apparatus which has been used to redetermine the rates of unfolding and refolding of ribonuclease A over a wide range of temperature and pH values. Under all conditions, the reaction is found to obey simple first order kinetics, suggesting that there is only one rate limiting step in the mechanism of unfolding, and refolding. On this assumption the rates of unfolding and

refolding can be determined. The rate of unfolding is found to increase exponentially with temperature, as expected for a simple process, while the rate of refolding increases with temperature below the melting temperature and decreases with increasing temperature above. This behavior is most simply explained by postulating a large increase in specific heat on formation of the denatured protein. [Dr. P. McPhie].

Serial No. NIAMD-LBM-1
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Carbohydrate Metabolism

Previous Serial Number: NIAMD-LBM-1

Principal Investigator: Dr. Gilbert Ashwell

Other Investigators: Dr. Jean Hickman Research Chemist
Dr. Lee Van Lenten, Staff Fellow
Dr. Stuart Snyder, Surgeon
Mr. William Pricer, Research Chemist

Cooperating Units: Dr. Anatol Morell, Department of Medicine,
Albert Einstein College of Medicine,
New York, New York

Man Years

Total:	6
Professional:	5
Other:	2

Project Description:

Objectives:

The major objective of this project has been a study of the role of the carbohydrate moiety of glycoproteins in determining the structure, function and metabolism of these macromolecules. Additional information is sought concerning the unique mechanisms of biosynthesis.

Methods Employed:

In general, the methods employed involve the use of highly purified enzymes to effect alteration or removal of specific monosaccharides on the protein. In some cases, radioactive labeling has been utilized to trace the rate of the modified molecule. Both in vivo and in vitro analyses are now carried out.

Major Findings:

In a continuing collaboration with Professor Anatol Morell at Albert Einstein College of Medicine, evidence has been obtained to extend the earlier observation that removal of terminal sialic acid residues from ceruloplasmin resulted in the rapid uptake of this protein by the liver. Upon injection into rats all of the desialylated proteins tested, with the exception of transferrin, were promptly removed from the circulation and were recovered in the liver. The materials examined included orosomucoid (α1 acid glycoprotein), fetuin, haptoglobin, α2-macroglobulin, lactoferrin, thyroglobulin, human chorionic gonadotropin and follicle-stimulating hormone. Competitive inhibition of hepatic uptake was demonstrated by the injection of tracer amounts of ^{64}Cu-ceruloplasmin together with substantive amounts of the above-mentioned desialylated proteins or the glycopeptides derived from them. Uptake was not inhibited by the fully sialylated protein or their glycopeptides. Employing the competitive inhibition technique, additional support was provided for the identification of the hepatocyte, and not the Kupffer cell, as the site of active accumulation of asialo--glycoproteins. [Dr. J. Hickman]

A general method has been described for the radioactive labeling of proteins containing terminal sialic acid in the carbohydrate moieties. Quantitative conversion of these residues to a radioactive 7-carbon analogue of sialic acid was activated by sequential periodate oxidation and borohydride reduction. The resulting radioactive preparations of several naturally occurring glycoproteins exhibited a normal half-life with plasma and proved suitable for metabolic studies. Application of this technique to human chorionic gonadotropin and follicle-stimulating hormone resulted in retention of biological activity. [Dr. L. Van Lenten]

The major locus of binding for desialylated glycoproteins has been identified as the hepatic plasma membranes and the binding process has been shown to involve a dual role for sialic acid. The presence of the latter on the membranes is essential whereas its presence on the glycoprotein is incompatable with binding. Complete reversibility was accomplished by alteration of the calcium ion concentration and pH of the medium thereby suggesting a possible model system for the binding and transport of proteins across the liver membranes. [Mr. W. Pricer]

Proposed Course of Project:

Current studies are in progress to develop a quantitative membrane assay which will permit the chemical identification of the glycopeptide moiety that is both necessary and sufficient for binding. Alternatively, the isolation and purification of the active binding site on the rat liver plasma membrane is being pursued as part of a program to elucidate the mechanism of glycoprotein transport across the liver cell wall.

Publications:

Van den Hamer, C. J. A., Morell, A. G., Scheinberg, I. H., Hickman, J., and Ashwell, G.: Physical and Chemical Studies on Ceruloplasmin IX. The role of galactosyl residues in the clearance of ceruloplasmin from the circulation. J. Biol. Chem. 245: 4397-4402, 1970.

Morell, A. G., Gregoriadis, G., Scheinberg, I. H., Hickman, J., and Ashwell, G.: The role of sialic acid in determining the survival of glycoproteins in the circulation. J. Biol. Chem. 246; 1461-1467, 1971.

Van Lenten, Lee, and Ashwell, G., Studies on the Chemical and Enzymatic Modification and Glycoproteins. A general method for the tritation of sialic acid-containing glycoproteins. J. Biol. Chem. 246: 1889-1894, 1971.

Beychok, S., Ashwell, G., Kabat, A., Optical Activity and Conformation of Carbohydrates III. Preparation and optical activity of methyl 2-acetamido-2-deoxy-α- and β-D-mannopyranosides and the corresponding furanosides. Carbohydrate Res. 17: 19-24, 1971.

Van Hall, E. V., Vaitukaitis, J. L., Ross, G. T., Hickman, J. W., and Ashwell, G.: Immunological and biological activity of HCG following progressive desialylation. Endocrinology 88: 456-464, 1971.

Serial No. NIAMD-LBM-2
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Enzymatic Utilization of Model Compounds

Previous Serial Number: NIAMD-LBM-2

Principal Investigator: Dr. William B. Jakoby

Other Investigators: Dr. Shelby L. Bradbury, Staff Fellow
Dr. Thorsten Fjellstedt, Sr. Asst. Scientist

Cooperating Units: None

Man Years

Total:	4
Professional:	3
Other:	1

Project Description:

Objectives:

These investigations are concerned with the reactivity of a variety of chemical groupings in enzyme-catalyzed reactions. Choices are such as to lead to investigations of the general phenomena of enzyme mechanisms. Particular attention is presently directed to a versatile detoxification mechanism by which addition compounds of glutathione are metabolized in preparation for excretion by the kidney.

Methods Employed:

The approach is that of purification of the chosen enzyme and its study by chemical and physical methods.

Major Findings:

These studies have continued a two-fold emphasis on the metabolic utilization of a variety of model compounds bearing specific functional groups and, as well, on those features of proteins responsible for specific catalysis.

Major Findings (continued):

In the former case are studies with epoxides which have resulted in the isolation of enzymes specific for the cleavage of such groups. [Dr. T. Fjellstedt] An enzyme from mammalian liver catalyzes the ring cleavage of both aliphatic and aromatic epoxides in the presence of glutathione and yields the thioether of glutathione and the epoxide. The thioether, in turn, is expected to be cleaved of its glycine and glutamic acid, two of the three amino acids which constitute glutathione, and then to be acylated at the site of the third amino acid, cysteine. The result is a group of compounds generically termed, mercapturic acids. Work with the glutathione-linked epoxidase has resulted in an enzyme preparation which is essentially homogenous. The purified preparation is being used in the determination of the physical and catalytic parameters of the enzyme.

Contributions in the latter group involve an extensive study of yeast aldehyde dehydrogenase [Dr. S. Bradbury] on enzyme composed of four similar or identical subunits. The catalytic reaction, i.e., conversion of aldehyde to acid, occurs after attachment to the protein of first aldehyde and then pyridine nucleotide. Inhibition of enzyme by one of the products of the reaction, reduced pyridine nucleotide, is being used as a means of approaching other aspects of the catalytic mechanism. One group of studies implicates a role for two sulfhydryl groups which are in close juxtaposition to each other.

Significance to NIAMD Research:

Studies of enzyme mechanism and of patterns of metabolism relate directly to the fundamentals of all biological activity. Work on epoxides is intimately related to a general mechanism of aromatic oxidation as well as to the biological problem of detoxification of noxious compounds.

Proposed Course of Project:

It is expected that investigations dealing with the above-noted projects will be continued and a study of other model reactions will be initiated.

Publications:

Clark, J. F. and Jakoby, W. B.: Yeast aldehyde dehydrogenase III. Preparation of three homogenous species. J. Biol. Chem. 245: 6065-6071, 1970.

Clark, J. F. and Jakoby, W. B.: Yeast aldehyde dehydrogenase IV. Dissociation and reassociation of native and hybrid species. J. Biol. Chem. 245: 6072-6077, 1970.

Packman, P. M. and Jakoby, W. B.: 5'-Phosphoribosyl-1-pyrophosphate. In Bergmeyer, H. U. (Ed.): Methoden der Enzymatische Analyse. Weinheim, Bergstr., Verlag Chemie, Vol. 2, 1970, pp. 1311-1313.

Kohn, L. D., Hurlbert, R. E., and Jakoby, W. B.: L(+)-and meso-tartrate. In Bergmeyer, H. U. (Ed.): Methoden der Enzymatische Analyse. Weinheim, Bergstr., Verlag Chemie, Vol. 2, 1970, pp. 1360-1366.

Scher, W. and Jakoby, W. B.: Maleat. In Bergmeyer, H. U. (Ed.): Methoden der Enzymatische Analyse. Weinheim, Bergstr., Verlag Chemie, Vol. 2, 1970, pp. 1584-1586.

Jakoby, W. B.: Oxalat. In Bergmeyer, H. U. (Ed.): Methoden der Enzymatische Analyse. Weinheim, Bergstr., Verlag Chemie, Vol. 2, 1970, pp. 1499-1502.

Jakoby, W. B.: Crystallization as a purification technique. In Jakoby, W. B. (Ed.): Methods in Enzymology. New York, Academic Press, Vol. 21, 1971, pp. 248-252.

Jakoby, W. B.: Enzyme purification and related techniques. Methods in Enzymology. New York, Academic Press, Vol. 21, 1971.

Bradbury, S. L. and Jakoby, W. B.: Ordered binding of substrates to yeast aldehyde dehydrogenase. J. Biol. Chem. 246: 1834-1840, 1971.

Jakoby, W. B. and Fjellstedt, T. A.: Epoxidases. In Boyer, P. D. (Ed.): The Enzymes. New York, Academic Press, Vol. 6, 3ed., in press.

Serial No. NIAMD-LBM-3
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Chemical and Enzymic Studies Related to the Structure, Metabolism and Function of Ribonucleic Acid

Previous Serial Number: NIAMD-LBM-3

Principal Investigator: Dr. Maxine F. Singer

Other Investigators: Dr. Carol Letendre, Staff Fellow
Dr. Janice Chou, Visiting Fellow
Dr. Randall K. Holmes, Research Associate

Cooperating Units: None

Man Years

Total: 4
Professional: 4
Other:

Project Description:

Objectives:

To continue studies on the mechanism of action of polynucleotide phosphorylase with special emphasis on the nature of the initiation reaction. To elucidate further the structure of the enzyme itself.

To study the mechanism and control of messenger RNA breakdown in Escherichia coli. This interest is being investigated in cells infected with bacteriophage T_7 since it has been established that the stability of messenger RNA is markedly increased upon T_7 infection.

Methods Employed:

To a large degree both our preparative and analytical work depends on the use of enzymes as specific reagents. The purely chemical techniques are of limited value with nucleic acids. Instead, a large inventory of highly specific enzymes is maintained. The enzymes are either prepared in the laboratory or in some cases, obtained commercially.

Methods Employed (continued):

Disk electrophoresis on polyacrylamide gels is used extensively both in mechanism studies and protein structure studies. Electrophoresis on acrylamide gels in the presence of sodium dodecylsulphate is also used.

Protein structure studies utilize the analytical ultracentrifuge and amino acid analyzer, as well as specific chemical and enzymic modification of the protein.

Experiments with bacteriophage T_7 use standard phage methods for growth, plating, isolation of mutants etc.

In addition standard biochemical, radiochemical and physical methods were used.

Major Findings:

1. Structural studies on polynucleotide phosphorylase. Probably the single most important accomplishment this year has been the introduction of chromatography on phosphocellulose for purification of polynucleotide phosphorylase. This has decreased to a large extent the time required to obtain pure enzyme and in addition, provides, for the first time, sufficient quantities of material to undertake detailed experiments in protein chemistry.

Additional evidence supporting the notion that the heterogeneity of pure, primer-independent polynucleotide phosphorylase (Form I) from Micrococcus luteus is the result of proteolysis during isolation has been obtained. The distribution and quantities of the various bands seen on disk electrophoresis varies from preparation to preparation. Nevertheless, the enzyme appears homogeneous in the ultracentrifuge suggesting that the various bands are approximately the same size. Under denaturing conditions, anywhere from 2 to 4 subunits are detected, depending on the preparation. Different bands of undenatured enzyme, isolated from disk electrophoresis gels, have a different distribution, both quantitatively and qualitatively, of the subunits. The single band of enzyme produced by limited tryptic hydrolysis (Form T) of the isolated enzyme has only one subunit. Thus, the structures of the original subunits are closely related and may indeed have been a single subunit prior to proteolysis during isolation.

Amino acid analysis of Form I, as well as titration of cysteine with ^{14}C-N-ethylmaleimide in reduced, denatured enzyme indicates that Form I has approximately 10 cysteine residues per molecule. Reduction of Form I followed by treatment with N-ethylmaleimide under mild denaturing conditions results in the addition of 3 moles of N-ethylmaleimide per molecule. Concomitant with this the enzyme becomes primer-dependent.

Major Findings (continued):

2. Mechanistic studies on polynucleotide phosphorylase. Extensive experiments using dADP and adenosine methylene diphosphonate (AOPCP) as inhibitors of the polymerization of ADP have yielded new insights into the mechanism of polynucleotide phosphorylase action. dADP is a powerful inhibitor of ADP polymerization at concentrations much lower than those of the ADP itself. With Form I it is a competitive inhibitor with a K_i of about 7 μM. Under the same conditions, AOPCP does not inhibit ADP polymerization at all. This is in marked contrast to the very similar effects of both dADP and AOPCP on oligonucleotide phosphorolysis. In the latter case both are competitive inhibitors with respect to P_i, with identical K_i values, namely 100 μM. Thus it appears that inhibition of polymerization by dADP involves a site on the enzyme other than that involved in oligomer phosphorolysis. It was proposed that in polymerization, the very sensitive inhibition is due to interference with initiation of de novo synthesis while the site with the higher K_i, which is sensitive to both dADP and AOPCP is the site involved in addition of monomers to the already growing chain. This interpretation is supported by the following additional findings. 1) With Form I, dADP specifically inhibits de novo polymer formation. 2) Addition of oligonucleotides overcomes dADP inhibition to a large extent and the reaction occurring is almost exclusively addition to oligomers. 3) Form T, which catalyzes the addition to oligomers more readily than does Form I is only very slightly sensitive to dADP inhibition in the presence of oligomer.

Albeit a strong inhibitor, dADP is also a substrate for polynucleotide phosphorylase. The enzyme catalyzes the exchange between the β-phosphate of dADP and inorganic ^{32}P. The exchange requires either oligomer or ADP and thus appears to proceed through intermediary steps of polymerization and phosphorolysis. Additional evidence against the alternative mechanism for exchange, namely formation of dAMP-enzyme complex, is derived from extensive but negative experiments attempting to detect such a complex.

That dADP can indeed take part in polymerization was demonstrated directly. A single dAMP residue is readily added to a preformed oligoribonucleotide primer. Further addition of dAMP or AMP is very difficult, indicating that the dAMP residue is a poor acceptor in the chain extension reaction. dAMP is also incorporated into polymer during de novo synthesis using a mixed substrate, of dADP and ADP. In this case however, dAMP residues occur at internal positions in the resulting copolymer. The polymers are of high molecular weight.

Mn^{2+} when it replaces Mg^{2+} as the required divalent cation in these reactions, always results in an improved utilization of dADP. Likewise, Mn^{2+} reverses to a large extent, the inhibitor of ADP polymerization by dADP.

Major Findings (continued):

3. Studies on messenger RNA degradation in E. coli. It has been reported that the messenger RNA produced by the bacteriophage T_7 genome in infected E. coli has a half life almost 10 times that of the messenger RNA in uninfected cells. In an effort to investigate the mechanism of this stabilization, all the known RNA degrading enzymes of E. coli were compared in infected and uninfected cells. No differences in the activity of any of the nucleases was detected.

One of the enzymes of interest is that called RNase V which has been implicated by others as the enzyme actually responsible for messenger RNA degradation. Following published and unpublished protocols by the workers first describing this enzyme we have been unable to reproduce their results. No nucleolytic activity with the reported properties could be detected. The nucleolytic activity measured under the stated conditions appears to have the properties of another known nuclease, RNase II. We have tentatively concluded that so-called RNase V is actually residual RNase II.

Significance to NIAMD Research:

The susceptibility of nucleotide polymerizing enzymes to limited proteolysis with concomitant alterations in activity has now been observed in several instances. Our work on polynucleotide phosphorylase therefore takes on a more general interest. It is unnecessary to detail the general importance of understanding the mechanism of biological polymerization reactions.

The instability of messenger RNA is central to many current ideas concerning biological control mechanisms. Nevertheless, little is known about the mechanism of messenger RNA degradation. One of the main accepted hypotheses in this regard, namely that so-called RNase V is responsible for messenger RNA degradation in E. coli is put in doubt by our inability to find any evidence for the existence of this enzyme.

Proposed Course of Project:

Investigation of the structure of polynucleotide phosphorylase will continue. The nature and position of functionally significant sulfhydryl groups will be investigated further.

We will also continue studies on the mechanism of stabilization of T_7 messenger RNA.

Publications:

Moses, R. E. and Singer, M. F.: Polynucleotide Phosphorylase of M. luteus. Studies on the polymerization reaction catalyzed by primer-dependent and primer-independent enzymes. J. Biol. Chem. 245: 2414-22, 1970.

Publications (continued):

Chou, J. Y. and Singer, M. F.: Synthesis of a copolymer containing adenylic and deoxyadenylic acid residues with polynucleotide phosphorylase. Biochem. Biophys. Res. Communs. 42: 306-311, 1971.

Serial No. NIAMD-LBM-4
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Naturally Occurring Sulfur Nucleotides: Identification, Chemistry, Biosynthesis and Biological Significance

Previous Serial Number: NIAMD-LBM-4

Principal Investigator: Dr. Marie N. Lipsett

Other Investigators: Dr. Jack Abrell, Research Chemist

Cooperating Units: None

Man Years

Total:	2.6
Professional:	2.0
Other:	.6

Project Description:

Objectives:

The sulfur transferase system which forms 4-thiouridine in tRNA is being studied because the ability of a tRNA to serve as an acceptor appears to reside not in its base sequence or secondary structure, but in its tertiary structure. Therefore the two enzymes which produce 4-thiouridine offer a tool for probing that tertiary structure. In addition, comparative studies with thiolated and unthiolated tRNA species can shed light on the function of 4-thiouridine in the tRNA molecule.

Dr. Jack Abrell has investigated conditions governing the release of ribonuclease I from E. coli, and has traced the apparent existence of two such enzymes to a procedural artifact.

Methods Employed:

Usual techniques of salt fractionation and column chromatography were used to purify the enzymes. Nucleotide digests were separated by electrophoresis and DEAE-cellulose chromatography. Standard spectrophotometric techniques were used for assays and kinetic studies on the ribonuclease.

Major Findings:

1. The first enzyme in the system has been purified 1000-fold, the sulfur-transferase itself 100-fold. Neither is yet homogeneous, but no other thionucleotides are formed beside 4-thiouridine. The only acceptor thus far found is tRNA. There appears to be no specific base sequence requirement, and no acceptor activity is found with synthetic polyribonucleotides (A, U, GU) or with multi-stranded complexes (A + U, A + U + U). Native or denatured DNA are likewise inactive. This specificity is probably a property of the first enzyme in the system, which "activates" the tRNA in preparation for the actual introduction of sulfur.

2. The second enzyme, the sulfur-transferase, contains pyridoxal phosphate. Inactivation by hydroxylamine can be reversed by pyridoxal phosphate, but not by pyridoxal, pyridoxamine or its phosphate. Inactivation also results from KBH_4 treatment (which reduces the PLP aldehyde or its Schiff base) or from incubation with amino acids (presumably through transamination to form the inactive PMP-enzyme). The apo-and holo-enzymes may be resolved on hydroxylapatite.

3. Despite the fact that yeast tRNA contains no 4-thiouridine, yeast extracts contain a sulfur-transferase activity which will substitute for that from E. coli, and will form 4-thiouridine in the presence of the first enzyme (coli), tRNA, and cofactors. This sulfur-transferase activity is also PLP-mediated.

4. Dr. Abrell has found that the release of Ribonuclease I is governed by the concentration of divalent cations in the growth medium of E. coli before osmotic shock. Two forms of Ribonuclease I, which differ in their chromatographic behavior in CG-50 resin, have been identified. One of these forms arises when the enzyme is treated with ammonium sulfate during its preparation, and the evidence indicates that in vivo there is only one form of Ribonuclease I.

Significance to NIAMD Research:

It is now possible to carry out a careful study of pure phenylalanine tRNA both in the native and enzymatically thiolated forms, and study the possible functions of 4-thiouridine in tRNA structure, in the amino acylation reaction, in ribosomal binding and in protein formation.

The first enzyme in the sulfur-transferase system, in selecting the thiolation site, offers a chance to explore the tertiary structure of tRNA, since it seems to be the tertiary structure rather than the immediate base sequence which governs the position of 4-thiouridine modification.

Proposed Course of Research:

The finding of PNP in the sulfur-transferase prompted a digression in the work while we explored this new facet. Consequently, many of the previous plans remain unchanged, and we are now ready to attack them.

Efforts to purify the sulfur-transferase to homogeneity will be continued, since relatively few enzymes in this class have been studied, and the PLP content of the enzyme increases its interest. Further studies on its mode of action will be carried out.

The nature of the actual sulfur-acceptor, the "activated tRNA" which is the product of the first enzyme in the system, will be further investigated, since the true site-specificity of the reaction may well lie at this step.

Pure yeast phenylalanine tRNA will be thiolated, and sequencing will determine whether the No. 8 uridine (the position which is invariably attacked in E. coli) is the site of substitution. Detailed work on the native and thiolated yeast $tRNA^{Phe}$ is planned to assess the differences in the physical or biological properties of the tRNA resulting from the presence of 4-thiouridine.

Dr. Abrell, during his remaining months at NIH, will study the kinetic properties of the two Ribonuclease I forms, as well as inhibitor and substrate specificities, and try to determine how the two forms differ.

Publications:

Abrell, J. W., Kaufman, E.E., and Lipsett, M. N.: The Biosynthesis of 4-Thiouridylate: Separation and purification of two enzymes in the transfer ribonucleic acid-sulfur-transferase system. J. Biol. Chem. 246: 294-301, 1971.

Abrell, J. W.: Ribonuclease I released from Escherichia coli by osmotic shock. Arch. Biochem. Biophys. 142: 693-700, 1971.

Serial No. NIAMD-LBM-5
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biosynthesis of Storage and Structural Polysaccharides and its Regulation

Previous Serial Number: NIAMD-LBM-5

Principal Investigator: Dr. Enrico Cabib

Other Investigators: Dr. Vladimir Farkas, Visiting Fellow
Dr. Kuo-ping Huang, Visiting Fellow

Cooperating Units: None

Man Years

Total: 4
Professional: 3
Other:

Project Description:

Objectives:

This project attempts to make use of polysaccharide biosynthesis, as a tool in the study of certain fundamental cellular processes, i.e., 1) the mechanisms by which morphological changes are initiated in the course of cell development, as exemplified by the formation of a septum in the yeast cell; 2) the regulatory systems for the biosynthesis of storage material, using glycogen in yeast as a model.

Methods Employed:

The organisms used are various strains on yeast. Mutants have been obtained in certain cases.

An effort is made to study each problem both at the molecular and at the cellular level. Thus, the usual methods of enzymology are employed to study the individual reactions involved in the process under investigation. At the same time, cells are incubated or grown under different conditions and submitted to a variety of determinations, such as direct observations under the light or electron microscope, assay of enzymes and of variations in their properties, and measurement of the changes in the concentration

Methods Employed (continued):

of certain metabolites. Radioactive isotopes are used both in the enzymatic assays and in studies of incorporation into different components of the intact cell.

Major Findings:

1. Earlier work showed that chitin is concentrated, as a disk-like structure, in the yeast bud scar, i.e. at the site in the cell wall where the bud has detached from the mother cell. The chitin disk is sandwiched between layers of glucan, a major component of the cell wall. Thus, chitin may be considered as the specific component of the yeast septum. Recently we have been able to show, with synchronously growing cells, that the synthesis of chitin only takes place during part of the cell cycle, namely between bud initiation and completion of the septum. Thus, the formation of chitin is initiated at a specific point in space and time. Newly discovered features of the enzymatic system concerned with chitin synthesis suggest how the triggering mechanism may operate.

When particulate preparations from Saccharomyces cerevisiae S2880 were submitted to mild sonic oscillation, the chitin synthetase activity was reduced to a very low value. Nevertheless, incubation of these particles with either the supernatant fluid from the sonic treatment, or with trypsin led to a 15 to 20-fold increase in activity. The activating factor present in the sonic supernate can be precipitated with ammonium sulfate and dialyzed. It is heat-labile and has therefore the characteristics of an enzyme. The heat-stable protein previously described by us as an inhibitor of chitin synthetase, has now been found to act as an inhibitor of the activating factor, which was contaminating our older preparations of the synthetase. Kinetic evidence indicates a very tight binding between the inhibitor and the activating factor.

A working hypothesis has been formulated, to explain how the three components of the chitin system, i.e. the synthetase, the activating factor and the inhibitor, may interact in the formation of the cell septum. The results indicate that chitin synthetase is present in the cell mainly in an inactive state, the "zymogen" state. It is suggested that the zymogen is distributed more or less uniformly on the cytoplasmic membrane. At the time of septum initiation, vesicles carrying the activating factor would be directed at the bud site. Thus the factor would transform zymogen into active enzyme at the desired location only. The function of the inhibitor is less clear, but its presence in the soluble fraction suggests that it could serve to trap any activating factor that might be released in the cytoplasm, thus preventing chitin synthetase activation at any other than the prescribed site.

Major Findings (continued):

2. Previous work of this laboratory showed that the transition from logarithmic to stationary phase of growth in yeast was accompanied by a dramatic increase in cell glycogen. The increase was correlated with a transformation of glycogen synthetase from the glucose-6-phosphate dependent into the independent form. This change did not occur in a mutant unable to accumulate glycogen. In order to understand the molecular mechanism underlying these changes, it was deemed necessary to purify and study the enzymes involved in glycogen synthesis, i.e. both forms of glycogen synthetase and the systems which interconvert one form into the other. As a beginning, the purification of glycogen synthetase in the D-form was undertaken. By a combination of ammonium sulfate precipitation, DEAE-cellulose and Sepharose 6B chromatography, a purification of several thousand-fold was obtained. Work is under way to scale up the preparation, in order to study the properties of the purified enzyme.

Significance to NIAMD Research:

The findings related to septum formation in yeast promise to provide for the first time the detailed molecular mechanism for the triggering of a morphological change in the cell. It is hoped that this case will provide a useful model for the general processes of differentiation and cell development.

The regulation of glycogen synthesis, on the other hand, is a model for the problem of energy distribution and storage in the cell. As was remarked in other occasions, there is a striking parallelism between these regulatory mechanisms, as observed in yeast and in mammalian cells. Furthermore, the possibilities of biochemical and genetic experimentation provided by a unicellular organism make of yeast a unique material for these studies.

Proposed Course of Project:

The discovery of the activating factor for chitin synthetase opens a number of avenues for future investigations. One of them is the purification of the activating factor and the elucidation of its enzymatic action. Another one is a study of the chitin synthetase zymogen, including its precise localization in the cell. A different approach will be to determine the variations of the different components of the system in synchronized cultures or in suitable mutants. Attempts will be made to explore these various paths, within the severe limitations in available manpower.

In the field of glycogen regulation, it is proposed to continue with the purification of the different enzymes from wild-type and mutant cells and with the study of their properties and interaction.

Publications:

Rothman-Denes, L. B. and Cabib, E.: Two forms of yeast glycogen synthetase and their role in glycogen accumulation. Proc. Nat. Acad. Sci. U.S. 66: 967-974, 1970.

Rothman-Denes, L. B. and Cabib, E.: Glucose 6-phosphate dependent and independent forms of yeast glycogen synthetase. Their properties and interconversions. Biochemistry 10: 1236-1242, 1971.

Cabib, E. and Bowers, B.: Chitin and Yeast Budding. Localization of chitin in yeast bud scars. J. Biol. Chem. 246: 152-159, 1971.

Keller, F. A. and Cabib, E.: Chitin and Yeast Budding. Properties of chitin synthetase from Saccharomyces carlsbegensis. J. Biol. Chem. 246: 160-166, 1971.

Cabib, E. and Keller, F. A.; Chitin and Yeast Budding. Allosteric inhibition of chitin synthetase by a heat-stable protein from yeast. J. Biol. Chem. 246: 167-173, 1971.

Serial No. NIAMD-LBM-6
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms

Previous Serial Number: NIAMD-LBM-6

Principal Investigator: Dr. Peter McPhie, Visiting Scientist

Other Investigator: Dr. Murray Summers, Staff Fellow

Man Years

Total:	2
Professional:	2
Other:	

Project Description:

Objectives:

Kinetic and thermodynamic studies on the denaturation and renaturation of ribonuclease A as a function of pH, temperature and solvent composition should give important information on the interactions within the molecule which direct the folding of the molecule into its active conformation from the denatured or newly synthetized state.

Kinetic studies on the binding of ethidium bromide to nucleic acids are hoped to reveal information on the process of intercalation, believed to be an important facet of dye-induced mutagenesis. By means of competition experiments, this system will be used to study the interaction of nucleic acids with other ligands, specifically tRNA with an aminoacyl tRNA synthetase.

Methods Employed:

Kinetic experiments on fast reactions notably the dye/DNA reaction are carried out in a combined "T-jump-stop-flow" device obtained from the American Instrument Company. Slower reactions such as the thermal unfolding of ribonuclease, which takes place over several seconds are being studied in a "slow T-jump" apparatus which has been developed with the aid of B.E.I.

Major Findings:

The slow T-jump device is finally working to specification and has been used to redetermine the rates of unfolding and refolding of ribonuclease A over a wide range of temperature and pH values. Under all conditions, the reaction is found to obey simple first order kinetics, suggesting that there is only one rate limiting step in the mechanism of unfolding, and refolding. On this assumption the rates of unfolding and refolding can be determined. The rate of unfolding is found to increase exponentially with temperature, as expected for a simple process, while the rate of refolding increases with temperature below the melting temperature and decreases with increasing temperature above. This behaviour is most simply explained by postulating a large increase in specific heat on formation of the denatured protein.

At any given temperature, the pH dependence of the rates of unfolding and refolding can be explained in terms of the titration of an important "trigger group" which transforms the neutral molecule, which unfolds slowly and refolds quickly into an acid form which unfold more quickly and refolds more slowly. Such an explanation has been suggested by a number of investigators for similar reactions. Application of the principle of microscopic reversibility to the system shows that, such a reaction scheme cannot apply (to this system, or to other investigators published data). A more reasonable explanation of the increasing rate of unfolding of the molecule is by decreased stability of the molecule as its net charge is increased by titration of all its carboxyl groups. The variation of the rate of unfolding can be predicted accurately by such a model. The variation of the rate of refolding is in the direction expected by such a model, but cannot be predicted with a similar degree of accuracy. This is probably because such a model takes no account of the change in specific heat discussed above. An alternative explanation is that refolding of the protein is more complex than we have thought and that the first order kinetics observed are actually steady state kinetics, hence the pH dependence of the rate of refolding is actually the pH dependence of a number of constants. Some experimental evidence for this has recently been obtained and is being actively persued.

Proposed Course of Study:

Kinetic studies of denaturation are being continued an synthetic derivatives of ribonuclease and on other proteins. Such comparative studies should give important information on the contributions made by different sections of the molecule and by different aspects of its primary and secondary structure to its stability.

A study has begun of the mechanism of phenylalanine tRNA synthetase of E. coli, with the aim of studying its interaction with its various substrates. These reactions can be monitored in the stop-flow and T-jump devices by coupling them with the dye-binding experiments and with other spectrophotometrically and spectrofluorometrically active ligands.

Serial Number NIAMD-LBM-7
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Hormone-Dependent Differentiation of Mammary Gland In Vitro

Previous Serial Number: NIAMD-LBM-7

Principal Investigator: Dr. Yale J. Topper

Other Investigators: Dr. Sylvia K. Friedberg, Dr. Takami Oka and Dr. Ida S. Owens

Cooperating Units: None

Man Years:

Total: 5
Professional: 4
Other: 1

Project Description:

Objectives - To study the molecular and cytological phenomena involved in development of mammary gland.

Methods Employed - Mammary gland explants are cultured on synthetic media. Synthesis of specific milk proteins is followed by isolation of these materials after the explants have been pulsed with radioactive precursors. DNA and RNA synthesis are determined in the same way. The activities of various enzymes related to DNA synthesis are assayed by conventional methods. In some instances explants are treated with collagenase to remove fat cells; the residual cells are then studied using methods cited above. Cell proliferation is studied by means of mitotic indices and autoradiography. Cytology is examined with the light microscope and electron microscope.

Major Findings - Insulin plays several key roles in the development of the mouse mammary gland, in vitro. It promotes epithelial cell proliferation, a process which appears to be necessary for differentiation. It plays an essential part, together with corticosteroid, in the formation of rough endoplasmic reticulum (RER). It also is required in order for prolactin to exert its functions on cells which had previously been exposed to both insulin and corticosteroid. Yet, in the intact virgin animal neither endogenous insulin nor corticosteroid exert these effects on the mammary epithelial cells. This, it turns out, is because the mammary cells in the non-pregnant animal are not sensitive to insulin. Insulin-sensitivity develops by the

second day of pregnancy. Furthermore, it appears that the elevation in the levels of corticosteroids which occurs shortly after the start of pregnancy is at least partly responsible for conferring insulin-sensitivity on the epithelial cells. This effect of corticosteroids has been demonstrated both in vivo and in vitro. Thus, corticosteroids make it possible for insulin to act, and insulin, in turn, makes it possible for the corticosteroids to participate in the formation of the RER.

Significance to Bio-medical Research and the Program of the Institute - During development of multicellular organisms the progenitors of many types of target cells which respond to different hormones are themselves not sensitive to the hormones. The system described above may be a useful model for studying developmental events which result in the conversion of hormone-insensitive into hormone-sensitive cells.

Proposed Course of Project - The molecular mechanism involved in the acquisition of insulin-sensitivity will be studied. Also, the reason why DNA synthesis is required for differentiation of mammary epithelial cells will be investigated.

Honors and Awards: None

Publications:

Green, M. R., and Topper, Y. J.: Some effects of prolactin, insulin and hydrocortisone on RNA synthesis by mouse mammary gland, in vitro. Biochim. Biophys. Acta 204: 441-448, 1970.

Topper, Y. J.: Multiple hormone interactions in the development of mammary gland in vitro. Recent Prog. Hormone Res. 26: 287-308, 1970.

Friedberg, S. K., Oka, T., and Topper, Y. J.: Development of insulin sensitivity by mouse mammary gland in vitro. Proc. Nat. Acad. Sci. U. S. 67: 1493-1500, 1970.

Topper, Y. J.: RNA-DNA: Some aspects of DNA synthesis in relation to differentiation. In Kretchmer, N. and Walcher, D. N. (Eds.). Environmental Influences on Genetic Expression: Biological and Behavioral Aspects of Sexual Differentiation. Washington, D. C., U. S. Govt. Printing Office, 1970, pp. 61-68.

Topper, Y. J., and Oka, T.: Steroids and the development of mammary epithelial cells. In Rabin, B. R. and Freedman, R. B. (Eds.). Proc. Symp. on Effects of Drugs on Cellular Control Mechanisms. (in press).

Green, M. R., Bunting, S. L., Topper, Y. J., and Peacock, A. C.: Changes in labeling pattern of RNA from mammary tissue as a result of hormone treatment. Biochemistry (in press).

Topper, Y. J., Friedberg, S. K., and Oka, T.: On the development of insulin sensitivity by mouse mammary gland in vitro. Proc. 29th Growth Symp., Soc. for Develop. Biol. (in press).

Serial Number NIAMD-LBM-8
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biosynthesis of Inositol in the Mammal

Previous Serial Number: NIAMD-LBM-8

Principal Investigator: Dr. Frank Eisenberg, Jr.

Other Investigator: Dr. Conrad Chen

Cooperating Units: None

Man Years:

Total: 1.5
Professional: 1.5
Other: 0

Project Description:

Objectives - The absolute requirement for NAD in the enzymatic isomerization of glucose 6-P to inositol 1-P suggests an intermediate oxidoreduction between substrate and product. Thus far, however, no intermediates, including NADH, have ever been demonstrated or isolated. Indirect evidence suggests that 5-ketoglucose 6-P is the cyclizing species, the reduction of inosose-P to inositol 1-P then accounting for the absence of NADH. Whether a single enzyme catalyzes this sequence of reactions is also unknown. An investigation into the obscure role of NAD in this reaction is now under way to ascertain if it performs as a cofactor for oxidoreduction. Establishment of that fact would support the intermediacy of 5-ketoglucose 6-P.

Methods Employed - Glucose 6-P labeled with tritium separately at C_5 and C_6 has been prepared by enzymatic phosphorylation of appropriately labeled glucose. Chemical degradation showed the expected labeling. NAD labeled with tritium in C_4 of the pyridine ring was prepared. Methods for the isolation and purification of nicotinamide, representing the oxidoreductive site of NAD, have been developed. Further purification of testicular cyclase is in progress. A new method for the gas chromatography of carbohydrates has been developed.

Major Findings - Because of its speed and sensitivity gas chromatography is an important analytical tool in this investigation. Although trimethylsilylation has been the method used for derivatization of sugars and cyclitols under study, its limitations have forced a search for better derivatives. A new reagent, n-butaneboronic acid, has been found which is superior in

several aspects. In an extension of the application of this reagent to carbohydrates, in general, it was found that the 3 biologically important hexoses, glucose, mannose, and galactose can be separated as alditol n-butaneboronates in less than 1/10 the time required for the alditol acetates; the silyl ethers of those same alditols cannot be separated. Another possible important use for this method is the quantitative analysis of glycoproteins for carbohydrate.

Significance to Biomedical Research and the Program of the Institute - The source of the enzyme system used in these studies is rat testis. Since little is known about the biochemistry of this tissue delineation of a new pathway of carbohydrate metabolism is in the interest of metabolic research.

An improved analytical procedure for carbohydrate will accelerate progress in the elucidation of glycoprotein structure, an area of great current interest.

Proposed Course of Project - NAD will be isolated from an incubation mixture consisting of purified rat testicular cyclase, NAD, and glucose, 5^3H 6-P or glucose, 6^3H 6-P and examined for radioactivity. If radioactive, then clearly the cofactor is involved in transfer of substrate hydrogen atoms via both C_4 positions of NADH; if not radioactive, the experiment is inconclusive since hydrogen transfer might still occur but involve the same position at C_4 in both stages. Conversely, starting with labeled NAD and unlabeled glucose 6-P, labeled product will indicate transfer at both positions; unlabeled product will be ambiguous since transfer of substrate hydrogen could go unrecognized if the same position of NADH is involved in both oxidation and reduction .

Adaptation of the gas chromatography of alditol n-butaneboronates to the quantitative analysis of glycoproteins for carbohydrate will be investigated.

Honors and Awards: None

Publications:

Eisenberg, F., Jr.: Relative stability of the interglycoside bonds of sucrose to acid hydrolysis in $H_2{}^{18}O$. Carbohydrate Res. 11: 521, 1969.

Eisenberg, F., Jr.: Inositol 1-phosphate. In Bergmeyer, H. U. (Ed.): Methods of Enzymatic Analysis. New York, Academic Press, (Ed. 3), (in press).

Eisenberg, F., Jr.: Inositol. In McGraw-Hill Encyclopedia of Science and Technology. New York, McGraw-Hill (1971 Revision) (in press).

Eisenberg, F., Jr.: Gas chromatography of phosphorus compounds. In Kolthoff, I.M., and Elving, P. J.: Phosphorus Analysis. New York, Interscience (in press).

Serial Number NIAMD-LBM-9
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Reductive Metabolism of Cystine in Cystinotic Cells

Previous Serial Number: NIAMD-LBM-9

Principal Investigator: Dr. Frank Tietze

Other Investigators: Dr. Joseph D. Schulman

Cooperating Units: None

Man Years:

Total: 1.3
Professional: 1.3
Other: 0

Project Description:

Objectives - To study the enzymatic reduction of the disulfide bond of the amino acid cystine in subcellular fractions of normal tissues and from individuals suffering from cystinosis.

Methods Employed - Cultured or freshly drawn leukocytes obtained from normal individuals and from cystinotic juvenile patients are disrupted and fractionated into subcellular components by differential centrifugation. These subcellular fractions are then examined for their ability to carry out the enzymatic reduction of cystine.

Major Findings - Whole cell extracts of normal human leukocytes are able to catalyze enzymatically the reduction of cystine, as well as other disulfide-containing substances, in the presence of reduced glutathione. The predominant proportion of this activity was found to be present in the soluble (cytoplasmic) fraction of the cell, with little or no activity detectable in the granular fractions. No difference in enzyme content as between normal and cystinotic cells was apparent in these studies.

Significance to Bio-medical Research and the Program of the Institute - Cystinosis is an inherited metabolic disorder characterized by an excessive and abnormal accumulation of the amino acid cystine within lysosomal granules of affected cells. Although the biochemical basis of this abnormality is not presently understood, it is reasonable to suppose, by analogy with other lysosomal storage diseases, that it is related to some defect in mechanisms

associated with the lysosomal metabolism or transport of the amino acid. It is evident that a rational therapeutic approach to this disease presupposes a further understanding of these processes in normal cells.

Proposed Course of Project - A further search will be made for indications of the existence of reductive or other degradative reactions undergone by cystine within lysosomes of normal mammalian cells.

Honors and Awards: None

Publications:

Tietze, F.: Disulfide reduction in rat liver. I. Evidence for the presence of non-specific nucleotide-dependent disulfide reductase and GSH-disulfide transhydrogenase activities in the high-speed supernatant fraction. Arch. Biochem. Biophys. 138: 177-188, 1970.

Tietze, F.: Disulfide reduction in rat liver. II. Chromatographic separation of nucleotide-dependent disulfide reductase and GSH-disulfide transhydrogenase activities of the high-speed supernatant fraction. Biochim. Biophys. Acta 220: 449-462, 1970.

Serial Number NIAMD-LBM-10
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Biochemical Lesions in the Genetic Mucopolysaccharidoses

Previous Serial Number: NIAMD-LBM-10

Principal Investigator: Dr. Elizabeth F. Neufeld

Other Investigators: Dr. Robert Barton, Dr. Michael Cantz, Dr. Jeffery Derge, Dr. Hans Kresse, Mrs. Clara Hall and Mr. James Scott

Cooperating Units: None

Man Years:

Total: 7
Professional: 6
Others: 1

Project Description:

Objectives - To determine the biochemical basis of the genetic disorders of mucopolysaccharide metabolism - i.e., the Hurler, Hunter and Sanfilippo syndromes, and related diseases.

Methods Employed - Fibroblasts derived from skin of normal individuals and affected patients are grown in tissue culture, and used to study the mucopolysaccharide metabolism in the genetic disorders as well as to assay the corrective factors. The factors, in turn, are obtained from normal human urine and purified by conventional biochemical techniques.

Major Findings - The abnormal mucopolysaccharide metabolism of fibroblasts from patients with the genetic mucopolysaccharidoses is due to the absence of a specific protein. When this protein is supplied exogenously, the metabolism is restored to normal. To date, five such proteins (designated "factors") have been observed: one deficient in the Hurler syndrome, one in the Hunter syndrome, two in the Sanfilippo syndrome (only one, however, in any one Sanfilippo patient), and one in the Maroteaux-Lamy syndrome. These factors are present not only in fibroblasts and fibroblast secretions, but also in normal human urine. In addition, fibroblasts from patients with an atypical mucopolysaccharidosis, which have no detectable β-glucuronidase activity, have an abnormal mucopolysaccharide metabolism correctible by β-glucuronidase. β-Glucuronidase can therefore be considered a sixth factor.

Urine was chosen as starting material for purification of the factors. A quantitative assay for the factors has been devised, and one unit defined as that amount giving half-maximal correction in one Petri dish of recipient cells under specified conditions. Purification methods have to take into account the lability of all factors at alkaline pH and at pH below 4, and the tendency of some factors to aggregate and lose activity at low ionic strength.

The purification of all the factors begins with concentration of proteins from 100 liter batches of pooled morning urine (kindly donated by the enlisted men at the Bethesda Naval Hospital) by precipitation with ammonium sulfate. The proteins, in concentrated solution, are applied to a 6 liter Sephadex G-200 column (courtesy of Dr. Maxine Singer); this separates the factors from each other, though there is some overlap. Subsequent steps differ for the Hurler, Hunter and Sanfilippo A factors (the three factors on which we have worked most). A procedure has been developed for the removal of albumin, a particularly bothersome contaminant in the Hunter factor. This involves passage through a column of anti-albumin linked to sepharose.

A thousand-fold purified Hurler factor has been shown devoid of activity toward all the other mucopolysaccharidoses. It is also devoid of most of the lysosomal enzymes which have high activity in the urine, but is still contaminated with β-N-acetyl hexosaminidase and acid phosphatase. It is estimated that at least another ten-fold purification would be required to obtain the factor in pure form, implying that it is but a trace constituent of urine.

Significance to Bio-medical Research and the Program of the Institute - The finding of specific protein deficiencies in the genetic mucopolysaccharidoses is of aid in the diagnosis and classification of these diseases, and offers prospects for rational therapy. One hopes that it will eventually be possible to treat patients, rather than just their fibroblasts, or, alternatively, to activate any defective factor that the patients themselves might be producing.

We are frequently asked to perform factor correction tests for diagnosis (primarily for genetic counseling) or prenatal diagnosis, by physicians caring for involved families.

The methodology developed is applicable to the study of other genetic disorders, as well as to non-genetic problems of connective tissue metabolism.

Proposed Course of Project - 1. An all-out effort will be made to work out the normal degradative pathway for the relevant mucopolysaccharides, dermatan sulfate and heparan monosulfate. It is assumed that the factors are enzymes along the degradative pathway, but their precise function cannot be guessed at in the present ignorance of the metabolic sequence involved.

2. Better sources of factors will be sought - e.g., in animal tissues or pathological urine - since the minuscule amounts present in normal urine preclude purification to homogeneity.

3. Antibodies to factors will be prepared, and fibroblast secretions or urine derived from affected individuals will be screened for possible cross-reacting proteins. Only by such means can one demonstrate conclusively that the factor deficiency is the primary effect of the mutation. If the hypothetical mutated factor were separable (e.g., electrophoretically) from the normal one, a reliable test for heterozygotes could be devised.

4. The Hurler factor may already be sufficiently pure and concentrated to warrant testing its effect on a patient. Permission to do so will be sought, and if it is granted, sufficient factor will be prepared to allow all the required preliminary tests for toxicity and pathogenicity to be performed. Procedures for monitoring anticipated effects on the patient will be set up.

Honors and Awards: None.

Publications:

Neufeld, E. F., and Fratantoni, J. C.: Inborn errors of mucopolysaccharide metabolism. Science 169: 141-146, 1970.

Wiesmann, U., and Neufeld, E. F.: Metabolism of sulfated mucopolysaccharide in cultured fibroblasts from cystic fibrosis patients. J. Pediatrics 77: 685-690, 1970.

Cantz, M. J., Chrambach, A., and Neufeld, E. F.: Characterization of the factor deficient in the Hunter syndrome by polyacrylamide gel electrophoresis. Biochem. Biophys. Res. Communs. 39: 936-942, 1970.

Kresse, H., Wiesmann, U., Cantz, M. J., Hall, C. W., and Neufeld, E. F.: Biochemical heterogeneity of the Sanfilippo syndrome: preliminary characterization of two deficient factors. Biochem. Biophys. Res. Communs. 42: 892-898, 1971.

Neufeld, E. F., and Cantz, M. J.: Corrective factors in the mucopolysaccharidoses. Trans. N. Y. Acad. Sci. (in press).

Neufeld, E. F.: The mucopolysaccharidoses. In Dorfman, A. (Ed.): Antenatal Diagnosis. Chicago, Univ. of Chicago Press (in press).

Serial Number NIAMD-LBM-11
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Enzyme Induction

Previous Serial Number: NIAMD-LBM-11

Principal Investigator: Dr. Irwin G. Leder

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 2
Professional: 1
Others: 1

Project Description:

Objectives - A. To study the inducible lac transport system in virus infected E. coli.

B. To study the synthesis and control of 4-methyl-β-hydroxymethyl thiazole in S. typhimurium.

Methods Employed - A. An overproduction of lac permease is induced in E. coli carrying a defective lac transducing phage. Derivatized polyacrylamide and polysaccharide adsorbants containing a specific thiogalactoside having a high affinity for the lac permease are used to selectively extract and purify the lac permease carrier protein.

B. Chemical mutagens are used to generate cells resistant to high concentrations of the thiamin antagonist "2-amino thiazole". Resistant strains producing an excess of thiamine thiazole are detected in cross-feeding experiments with thiazole requiring mutants of the same test organism.

Major Findings - A. The heptaacetyl derivative, methyl (2,3,4,6 tetra-O-acetyl β galactopyranosyl 2,3,4 tri-O-acetyl-1-thio β-galactopyranoside) uronate has been synthesized and characterized by elementary, O-acetyl and NMR analysis. Deacetylation has yielded a crystalline ester, not yet characterized, which has a high affinity for the lac permease.

B. Approximately 20 mutants resistant to 0.01 M "2-aminothiazole" have been isolated. Several of these appear capable of cross feeding a thiazole requiring test strain.

Significance to Bio-medical Research and the Program of the Institute - A. Elucidation of the role, structure and integration of the *lac* carrier protein in the bacterial membrane will contribute to our understanding of the properties of similar membranous structures in eucaryotic cells.

B. The mechanism of "thiazole" synthesis is unknown. This study will add to our understanding of the intermediary metabolism of sulfur containing amino acids.

Proposed Course of Project - A. The structure of the deacetylated thiogalacturonide will be established and the free acid prepared and coupled to suitable adsorbants. Extracts from virus infected and *lac* induced cells will be tested for the presence of proteins which are bound to the modified adsorbants and which may be specifically displaced by substrates of *lac* permease.

B. Mutants of *S. typhimurium* which appear to cross-feed thiazole requiring cells will be grown in synthetic liquid medium and the cell free medium examined for components which stimulate the growth of test organisms. Bioautographic techniques will be used to identify growth factors capable of replacing free "thiazole".

Honors and Awards: None

Publications: None

Serial Number NIAMD-LBM-12
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Synthesis and Secretion of Immunoglobulins

Previous Serial Number: NIAMD-LBM-12

Principal Investigator: Dr. Milton Kern

Other Investigators: Dr. Earl W. Sutherland III and Dr. Daniel H. Zimmerman

Cooperating Units: None

Man Years:

Total: 3.5
Professional: 3
Others: .5

Project Description:

Objectives - The purpose of this project is to study the nature of the intracellular mechanisms responsible for the synthesis and secretion of immunoglobulins.

Methods Employed - Immunoglobulin producing cells, incubated with ^{3}H-galactose, ^{3}H-glucosamine, ^{3}H-mannose or ^{3}H-leucine, were lysed with deoxycholate in the presence of iodoacetamide to preclude further disulfide bond formation. The specifically precipitable immunoglobulin components derived from murine myeloma (ADJ-PC5) and popliteal lymph node cells were then separated by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The intracellular distribution of such components in rough and smooth microsomes and cell sap fractions was also examined.

Major Findings - Murine myeloma cells (ADJ-PC5), incubated *in vitro* with ^{3}H-leucine, secrete ^{3}H-immunoglobulin G (IgG) as a single molecular species as judged by the migration characteristics of the labeled product on sodium dodecyl sulfate-acrylamide gel electrophoresis. However, the fact that some of the interchain disulfide linkages of intracellular immunoglobulins had not been acquired permitted the identification of the following intracellular species: LHHL (identical to IgG), HHL, HH and L (H and L refer to heavy and light polypeptide chains, respectively). Although HH and HHL were readily observed, radioactivity was not detected in the region of the gel where HL would be expected. The smooth microsome fraction contained principally LHHL, while HHL and HH were the predominant components in the

rough microsome fraction. Only LHHL was labeled when cells were incubated with ^{3}H-galactose and such immunoglobulins were found mainly in the smooth fraction. On the other hand, immunoglobulins labeled with ^{3}H-mannose and ^{3}H-glucosamine were confined principally to the rough microsomes and the label appeared not only in LHHL but in HH and HHL as well. These findings indicate that some sugar residues are acquired before the synthesis of certain of the disulfide bonds. Moreover, galactose appears to be acquired at a site distinguished from the intracellular site where some glucosamine and mannose residues are added to the partially synthesized immunoglobulin molecule.

Significance to Bio-medical Research and the Program of the Institute - The abnormal γ-globulins and their subunits that have been observed in serum and urine may be a consequence of aberrations in the secretory mechanism, as well as the synthetic mechanism. Studies from this laboratory have elucidated several new parameters that define synthesis and secretion of normal γ-globulin and provide a means for assessing the similarities and dissimilarities between a normal and abnormal cell. In addition, such parameters may also be useful in distinguishing whether the differentiation of lymphocytes to yield cells which produce and secrete γ-globulin develop the polypeptide synthesizing and secretory apparatus simultaneously or not.

Proposed Course of Project - Efforts will be directed toward further elucidation of the intracellular compartments involved in secretion. The role of the Golgi apparatus will be investigated using newly reported techniques for its isolation from cell homogenates. In addition, characterization studies of secretory colostral IGA and the IGA product derived from rabbit appendix cells will be continued.

Honors and Awards: None

Publications:

D'Amico, R. P., and Kern, M.: Synthesis and secretion of γ-globulin by lymph node cells. VII. The cell-free incorporation of galactose and sialic acid into the carbohydrate component of endogenous and exogenous immunoglobulin. Biochim. Biophys. Acta 215: 78-87, 1970.

Sutherland, E. W. III, Zimmerman, D. H., and Kern, M.: Synthesis and secretion of lymph node cells. VIII. The order of synthesis of the interchain disulfide linkages of immunoglobulins. Proc. Nat. Acad. Sci. U. S. 66: 987-994, 1970.

Kern, M.: The role of carbohydrate in immunoglobulin secretion. In Jamieson, G. J. (Ed.): Glycoproteins of Blood Cells and Plasma. New York, J. B. Lippincott (in press).

Serial Number NIAMD-LBM-13
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Investigation of a Formal Complaint of Racial Discrimination for the NIH Equal Empolyment Opportunity Board.

Previous Serial Number: None

Principal Investigator: Dr. Frank Eisenberg, Jr.

Other Investigators: None

Cooperating Units: NIH Equal Employment Opportunity Board

Man Years:

Total: .4
Professional: .4
Other: 0

Project Description: Project title is self-explanatory.

Honors and Awards: None

Publications: None

SECTION ON BIOCHEMICAL MECHANISMS

Stereopopulation Control in Simulation of Transpeptidation

A model for enzyme-catalyzed transpeptidation had previously been developed by study of lactam formation from o-aminophenoxyacetylglycylglycine. By introduction of conformational restriction as a means of bringing the peptide bond and nucleophile into juxtaposition in the ground state, the rate of reaction is increased 3500-fold. In the improved model, the half-life for transpeptidation at 30° and pH 3.5 has been decreased to 15 seconds. Models are being devised to approach enzymatic rates even more closely (L. A. Cohen and K. L. Kirk).

Stereopopulation Control in Simulation of Ester Formation

By use of appropriately substituted phenolic acids and freezing of rotation of single bonds, rates of lactonization have been increased to a point at which the half-life for reaction is reduced to 0.2 seconds at pH 6 and 30°. So effective is the rotational freezing that lactonization is still going on at pH 11-12. The rate for the most reactive compound to date exceeds that for phenol with acetic acid by a factor of 10^{15}. Additional models are being prepared in which rate enhancement is expected to reach 10^{20} (L. A. Cohen and S. Milstien).

Stereopopulation Control in Simulation of Enzymatic Displacement Reactions

An intramolecular model for methyl transfer has been synthesized in which a carbon-oxygen bond is created by attack of a phenol on an alkyl sulfonate. By introduction of conformational restriction, the half-life for reaction at pH 7 and 30° is reduced to 3 seconds. The most reactive compound is faster than the phenol-methyl sulfonate pair by a factor of 10^{11}. Failure to achieve the same effect with methylsulfonium species emphasizes the rigid orientational requirements of a nucleophilic displacement reaction (L. A. Cohen and R. T. Borchardt).

Stereopopulation Control in Simulation of Fumarase Action

In a molecule containing a suitably activated double bond (benzoquinone) and a conformationally restricted hydroxyl group, addition of the nucleophile occurs rapidly at pH 8, simulating the addition of water to fumarate to form malate. Since the same reaction cannot be detected with less restricted models or with benzoquinone and an alcohol, the minimum estimate for a rate enhancement factor is set at 10^{10} (L. A. Cohen and R. T. Borchardt).

Stereopopulation Control in a Test of the Mitchell Hypothesis

A homolog of imidazolepropionic acid was devised with conformational

control built in so that spontaneous cyclization to an acylimidazole would occur. It was postulated that a sudden drop in pH, with protonation of the imidazole ring, would effect the generation of acyl phosphate in the presence of inorganic phosphate. Synthesis of the model compound has been beset with various obstacles, but is now essentially complete, and kinetic studies will soon be initiated (L. A. Cohen and Y. Kikugawa).

Stereopopulation Control in a Simulation of Aldehyde Dehydrogenase

Benzoquinone containing a conformationally restricted propionaldehyde side chain was found to undergo hydride transfer in both solution and solid state. The hydrated aldehyde serves to reduce the quinone to hydroquinone, itself undergoing oxidation to acid, ester or thiolester, according to the medium. This reaction represents the first effective model for the formation of thiolester by oxidation of a hemithioacetal with NAD^+ (L. A. Cohen and R. T. Borchardt).

Conformation and Resonance Coupling in Phenolic Esters

Because of the implication of hydroquinone esters in oxidative phosphorylation, the physical and spectral properties of phenolic esters in general have been a matter of concern. Carboxylic esters of phenols are not coplanar molecules because the alkyl group of the acid portion is crowded by the ortho hydrogens of the benzene ring. An angle of twist of 60° out of plane is postulated. Resonance overlap between the phenolic oxygen and the benzene ring is disrupted, as evidenced by the correlation of carbonyl frequency with σ_I (para) rather than with σ_T (para). This effect is not due to out-of-plane rotation but to competition for the phenolic oxygen by both benzene ring and carbonyl; the ester resonance is more important than phenolic resonance. This conclusion was reached by studying phenyl formates, in which there is no twist out of plane and still a correlation with σ_I alone (L. A. Cohen and Y. Kikugawa).

Transient Species in Imidazole Chemistry

When imidazolediazonium species are decomposed thermally or photochemically, intermediate imidazolecarbenes are formed. Thus, when imidazole-4-diazonium-5-carboxylic ester is heated in glacial acetic acid, imidazole-5-carboxylic ester is obtained, the solvent providing either a hydrogen atom or a hydride ion. The transfer cannot involve a proton, since the compound is completely stable in trifluoroacetic acid. Similarly, photolysis of the diazonium compound in benzene solution provides the corresponding phenyl-substituted imidazole. Whether the carbene is of the singlet or triplet type (or both) is still uncertain.

Photolysis of imidazole-4-diazonium-5-carboxylate leads to evolution of carbon dioxide and nitrogen, and formation of an imidazyne (analogous to benzyne). The properties of these systems are being investigated further. This latter system is of particular interest because of the antimitotic properties of the corresponding carboxamide (L. A. Cohen and K. L. Kirk).

Photochemical Synthesis of Fluorinated Imidazoles

By photolysis of imidazolediazonium fluoborates in fluoboric acid solution, nitrogen is evolved and fluoroimidazoles are obtained for the first time. Compounds already prepared in this way include 2-fluoroimidazole, 4-fluoroimidazole, 4-fluoroimidazole-5-carboxamide and fluoro-DL-histidine. The latter amino acid is bound very tightly to histidine deaminase and is slowly deaminated. The compound also shows an hypnotic or soporific effect in mice. Fluorine analogs of purine precursors are being synthesized with a view to inhibition of polynucleotide synthesis or incorporation in the polynucleotide chain (L. A. Cohen and K. L. Kirk).

SECTION ON CARBOHYDRATES

The Oxidation of 2-Acetamido-2-deoxyaldoses

While the available biochemical evidence appears to indicate fairly convincingly that 2-acetamido-2-deoxy-D-glucono-1,5-lactone is the most effective of the known inhibitors of epididymal β-N-acetyl-D-glucosaminidase, the substance itself has received scant attention; the compound has not been characterized adequately nor has a satisfactory method for its preparation been described. Indeed, no closely related structures appear to have been reported in the literature and the oxidation processes which are so useful in making aldonolactones from ordinary aldoses have apparently not been applied to the 2-acylamido-2-deoxy aldoses. For these reasons, a study of the oxidation of the more readily accessible 2-acylamido-2-deoxyaldohexoses has been initiated.

With unbuffered bromine water, 2-acetamido-2-deoxy-D-galactose and -D-mannose give the corresponding 2-acetamido-2-deoxy-hexono-1,4-lactones which are isolable in crystalline form and in substantial yield. 2-Acetamido-2-deoxy-D-glucose, on the other hand, affords mixture which contains 2-amino-2-deoxy-D-gluconic acid, 2-acetamido-2-deoxy-D-gluconic acid, 2-acetamido-2-deoxy-D-glucono-1,5-lactone and another, as yet unidentified lactone. 2-Acetamido-2-deoxy-D-gluconic acid may be isolated from the mixture as its crystalline dicyclohexylammonium salt. The mother liquor contains 2-acetamido-2-deoxy-D-mannono-1,4-lactone, and it is likely that the isomerization involved in the formation of this compound is brought about by the alkalinity of dicyclohexylamine. Treatment with dimethylamine of the crude mixture from the oxidation of 2-acetamido-2-deoxy-D-glucose gives 2-acetamido-2-deoxy-di-N-methyl-D-mannonamide; here again a facile conversion from the D-gluco to the D-manno configuration has taken place.

When treated with methanolic alkali, 2-acetamido-2-deoxy-D-mannono-1,4-lactone, 2-acetamido-2-deoxy-D-galactone-1,4-lactone and the mixture obtained by the oxidation of 2-acetamido-2-deoxy-D-glucose all give the same mixture of two unsaturated lactones. These have been shown to be the 2-acetamido-2,3-dideoxy-D-hex-2-enono-1,4-lactones of the erythro and threo configurations; they

are interconvertible under alkaline conditions. Their formation is reminiscent of the elimination shown by O-glycosides of threonine and serine moieties in glycoproteins.

The 2-Acetamido-2-deoxyhexoses that were the object of the investigation described above may exist in either the pyranose or furanose ring forms. Attention has been given to the oxidation of 2-acetamido-2-deoxy-D-glucose derivatives that are locked into the pyranose form by suitable substitution. Aside from the general objective of widening knowledge of the chemistry of the aminosugars, this work was designed to afford 2-acetamido-2-deoxyaldonic acid derivatives of unequivocal structure and configuration. With methyl sulfoxide-acetic anhydride as an oxidant, 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-D-glucopyranose gives 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-D-glucono-1,5-lactone in crystalline form and in very high yield; removal of the benzyl groups from this lactone affords 2-acetamido-2-deoxy-D-glucono-1,5-lactone and this pathway constitutes the first unequivocal and reproducible synthesis of this biochemically interesting substance. With dimethylamine, 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-D-glucono-1,5-lactone gives 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-di-N-methyl-D-gluconamide but the amine partially rearranges this product into the corresponding D-mannoamide, showing that the gluco-to-manno rearrangement observed earlier may take place with acyclic derivatives.

2-Acetamido-3,4,6,-tri-O-acetyl-2-deoxy-D-glucopyranose and 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-D-mannopyranose are oxidized by methylsulfoxide-acetic anhydride to the corresponding 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-D-hexono-1,5-lactones. In each case, however, a substantial quantity of 2-acetamido-4,6-di-O-acetyl-2,3-dideoxy-D-erythro-hex-2-enono-1,5-lactone was formed, showing that β eliminations can readily take place in these six membered lactones when an effective leaving group is present.

From the foregoing it is evident that 2-acetamido-2-deoxyaldonic acids have little in common with their better known, non-nitrogenous analogs. (N. Pravdić and H. G. Fletcher, Jr.)

Unsaturated Aminosugars

Earlier work in this Section led to the discovery of a new type of carbohydrate, unsaturated aminosugars typified by 2-acetamido-D-glucal (2-acetamido-1,2-dideoxy-D-arabino-hex-1-enopyranose). This particular substrate, incidentally, has been found to be a mild inhibitor of N-acetyl-D-glucosaminidase. By way of exploring the potential of compounds of this class for synthetic purposes, the behavior of 3,4,6-tri-O-acetyl-2-(N-acetylacetamido)-1,2-dideoxy-D-arabino-hex-1-enopyranose with various acids and phenols in the presence of an acidic catalyst was studied. Evidence was obtained which indicates that the unsaturated aminosugar derivative initially rearranges under the reaction conditions used,

the acyloxy group at C-3 migrating to C-1 and the double bond shifting to the C-2-C-3 position. Following this allylic shift, the nucleophile provided (carboxylic acid or phenol) displaces the acetoxy group at C-1, giving an ester or glycoside of 2-acetamido-2,3-dideoxy-D-erythro-hex-2-enopyranose. This facile reaction opens up wide-ranging synthetic possibilities (N. Pravdic and H. G. Fletcher, Jr.).

Synthesis with Partially Benzylated Sugars

The synthesis of 1,3,4,6-tetra-O-benzyl-D-fructofuranose in this Section was described in an earlier report. Exploration of the utility of this substance for various synthetic objectives has now been initiated. While the compound appears to be readily convertible into 1,3,4,6-tetra-O-benzyl-D-fructofuranosyl chloride, the value of this halide for the introduction of fructofuranosyl moieties into various structures may be questioned inasmuch as the substance readily undergoes dehydrohalogenation, giving two new unsaturated ketose derivatives that were shown to be 1,3,4,6-tetra-O-benzyl-2-deoxy-D-erythro-hex-2-enulofuranose and 1,3,4,6-tetra-O-benzyl-2-deoxy-D-arabino-hex-1-enulofuranose. (E. Zissis, R. K. Ness and H. G. Fletcher, Jr.).

In another context, the condensation of 1,3,4,6-tetra-O-benzyl-D-fructofuranose with 2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl chloride was found to yield octa-O-benzylsucrose from which the benzyl groups were readily removed by catalytic hydrogenolysis. The octa-O-benzyl ether of α-D-glucopyranosyl α-D-fructofuranoside was also formed in this condensation and was found to be a product in the condensation of 1,3,4,6-tetra-O-benzyl-D-fructofuranose with 2,3,4,6-tetra-O-benzyl-D-glucopyranose in the presence of perchloric acid. (R. K. Ness, H. G. Fletcher, Jr.)

A highly novel method for the cleavage of carbohydrate benzyl ethers has been discovered. The active reagent consists of a mixture of a thiol and boron trifluoride etherate. If the carbohydrate liberated is capable of reacting with the thiol, it will do so. Thus when 1,2-ethanedithiol is used, 2,3,4,6-tetra-O-benzyl-D-glucopyranose gives D-glucose ethylene dithioacetal while ethanethiol gives a mixture of the anomeric ethyl 1-thio-D-glucopyranosides. The fate of the benzyl groups also depends upon the thiol used. With 1,2-ethanedithiol one obtains a mixture of benzyl sulfide and tribenzylsulfonium tetrafluoroborate; with ethanethiol, the product is dibenzyl ethyl sulfonium tetrafluoroborate. This dibenzylation process has been developed into a method for the identification of the amorphous anti-inflammatory drug "Glyvenol" (ethyl 3,5,6-tri-O-benzyl-D-glucofuranoside).
(H. G. Fletcher, Jr. and H. W. Diehl)

Immunochemistry

As we reported last year, attempts were under way to prepare capsular polysaccharide from Type 61 pneumococcus. We have grown

and killed preparative batches by a number of different ways and the various batches isolated are being purified by Sephadex column chromatography. An obstinate impurity in all our preparations is species specific C-substance antigen, and we have found that the only way to remove it is by precipitation with TEPC 15 myeloma anti-C serum (M. Potter) followed by DEAE-cellulose chromatography of the supernatant solution to obtain the capsular polysaccharide.

Synthesis of Indolyl Galactosides

The diagnosis of Fabry's disease (a form of sphingo-lipidosis) requires the preparation of an indolyl α-D-galactopyranoside to show the presence or absence of an α-galactosidase in tissue. This is a 1,2-*cis* glycoside and its synthesis is sufficiently difficult that it has as yet not been successfully reported in the literature. We are presently pursuing its preparation.

Oxidation of Glycosides

The chromium trioxide acetic acid oxidation of glycosides has been investigated following reports on the oxidation of alkyl ethers. There is hope that the reaction will lead to a way of selectively oxidizing 1→6 beta linkages in oligo- and polysaccharides (C. P. J. Glaudemans).

Higher-Carbon Sugars and their Derivatives

Reinvestigation of a fraction of an avocado extract has shown that that syrup, originally believed to contain D-*talo*-heptulose, contains both a *talo*-heptulose and an *allo*-heptulose. Syrupy D-*threo*-L-*ido*-octose has been converted, by long standing, into D-*glycero*-L-*gluco*-octulose. The same crystalline octulose was also obtained by oxidation of D-*threo*-L-*gulo*-octitol with *Acetobacter suboxydans*. Treatment of the octulose with methanol and an acid catalyst produced crystalline methyl D-glycero-α-L-*gluco*-octulopyranoside, whose structure was proved by degradation to methyl α-L-*gluco*-heptulopyranoside (N. K. Richtmyer).

SECTION ON MEDICINAL CHEMISTRY

Polymeric Antimetabolites

Poly-L-tyrosine has shown pronounced inhibition of collagenase implicating tyrosine in the site of attack of this enzyme on collagen. Chemical studies aimed at developing better polymeric carriers have lead to the synthesis of 4-(p-amino or p-hydroxy)phenyl-1-vinylpyrrolidone for copolymerization to a potential carrier (T. D. Perrine).

Instrumentation

A mouse "shocker" and dispenser for use in testing for analgesic activity have been developed and perfected. In addition the automatic viscometer developed in this laboratory has been refined to a precision of one part in 10^5. Judging from preliminary studies on the mutarotation of glucose, this viscometer will have utility in other kinetic studies (T. D. Perrine).

Computer Analysis in Chemical Structure-Biological Activity Correlations

Substituent pi values determined from several partition coefficients in the benzomorphan series and which are indicative of transport of the compounds concerned to a presumed active site were combined with interatomic steric factors (to simulate interference with metabolic N-demethylation) to give a series of equations. These equations (one equation/compound) were subjected to computerized regression analysis. The final equations will be used in subsequent synthetic work toward improved benzomorphan-like drugs (A. E. Jacobson).

Benzomorphan Research

A new, versatile unambiguous synthesis of 1,2,5,6-tetrahydropyridine precursors of pharmacologically important 6,7-benzomorphans has been developed from appropriately substituted 2-bromopyridines. This sequence makes available hitherto inaccessible benzomorphans and is applicable to a reduced-cost synthesis of the medically useful and narcotically uncontrolled pentazocine (G. Thyagarajan).

Antiinflammatory Compounds

1,4-Bisphenethylamino-2-aminobutane and the methylamino analog were more effective than oxyphenbutazone in inhibiting exudate formation in the croton-oil granuloma pouch technique. Corresponding piperidine compounds were equal to indomethacin (E. L. May).

Asparagine Synthetase and Tumor Inhibitors

For comparison with the L-aspartic acid series where 1,4-bis-2-phenethylamino-2-aminobutane and its N-methyl derivative caused 90-100% *in vitro* inhibition of both asparaginase-sensitive and asparaginase-resistant leukemics, corresponding substances of the D-series along with piperidino analogs of both the L- and D-series have been made for assessment against various synthetases and tumors. Test results are not complete (J. H. Ager and E. L. May).

Novel Ring Systems via Photocyclizations and the Pictet-Spengler Reaction

The photocyclization method of Witkop, *et al.*, has been extended to complex systems with fixed steric configuration. For example, *cis* (a)-9-amino-5-(*m*-methoxyphenyl)-2-methyl-2-azabicyclo[3.3.1]nonane (I) prepared from the corresponding 9-ketoxime (Na/C_2H_5OH gave 70% of the *trans*-amine), was O-demethylated, then selectively N-chloroacelylated and the product photolyzed to give a mixture which, after separation into two main products, CH_2N_2 treatment, diborane reduction, and O-demethylation of each gave 10-hydroxy-4,11b-iminoethano-12-methyl-1,2,3,4,4a,6,7,11b-octahydro-5H-dibenz[b,d]azepine and the 8-hydroxy isomer. Their 5-methyl homologs were also prepared by CH_2O/HCO_2H treatment prior to O-demethylation. This appears to be the first example of *ortho* ring closure in photocyclizations of this type.

Pictet-Spengler cyclization of I gave 9-methoxy-4,10b-iminoethano-1,2,3,4,4a,5,6,10b-octahydrobenz[c]quinoline which was demethylated to the 9-hydroxy relative. Again the 5(N)-methyl homolog was prepared prior to O-demethylation.

These novel complex systems are being biologically evaluated (H. H. Ong and E. L. May).

Synthetic Methods for Alkaloids

Model syntheses for alkaloids starting from appropriate dinitriles and proceeding through iminoamines (as the penultimate compounds) have been successful. Some of these involving indolizine amines as intermediates appear to point the way to the preparation of such complex alkaloids as camptothecin, a clinically active antitumor agent available in limited supply from natural sources only (E. M. Fry).

Codeinone Studies

Codeinone and excess dimethyloxosulfonium methylide gave instead of the expected 6,7-fused cyclopropyl derivative, unchanged material and a new substance, separated by careful column chromatography, which appears from spectral data to be a new 6-spirooxirano compound with twice the analgesic potency of codeine (L. J. Sargent).

Aldolase Inhibitors

Using the method of oxidative phosphonylation described a year ago, refinements in the preparation of dihydroxyacetone-O-phosphonate, glyceraldehyde-O-phosphonate and fructose di-O-phosphonate (all isolated as water soluble, neutral Mg salts) have been made. Also two alternate routes to glucuronides (improvements over the tedious Helferich and Koenigs-Knorr synthesis) have been found. This is of interest because recently it has been reported that glucuronides can selectively block metabolic processes. Evaluation of the glucuronides and phosphonates is in progress. Finally, reaction of 2,5-dihydroxybenzocic acid with resorcinol gave a yellow product which is almost certain to be 2-(2',2'-dihydroxybenzoyl)-1,4-benzoquinone, rigidified by hydrogen bonding. Because of its analogies to the salicylates it may have anti-inflammatory properties (J. G. Murphy).

Testing for Analgesic Activity and Dependence Liability

In the assessment of 87 new compounds (mostly from the pharmaceutical industry and universities) for analgesic activity, 40 were of sufficient interest to justify their study for morphine-like dependence liability. In addition four were tested at the Southern Research Institute for barbiturate-like dependence. Two were studied in man.

The Nilsen test for analgesic activity, recently installed in our laboratory with improvements in instrumentation and methodology, continues to be a valuable complement to the regularly used hot-plate technique. This is particularly so, as the major thrust in this area of research is on antagonist-type analgesics; the Nilsen method is of better predictive value for these types (E. L. May, L. J. Sargent and N. B. Eddy).

United Nations Activities

Examination of several cannabis samples revealed only traces of 3-5 alkaloids which appear to be of minor or no importance in the psychotropic activity of Cannabis. Improved extraction methods and solvent systems in chromatography were developed during four months of experimentation toward a more comprehensive analysis of this plant including genetic, climate, soil, time-of-harvesting and geographic factors. During experiments in quantitative gas chromatography, it was shown that certain psychotropically inactive components were decarboxylated and/or ring-closed to tetrahydrocannabinols and other cannabinoids which may explain why some samples, although containing no tetrahydrocannabinol, are active on ingestion by smoking.

One month was served as consultant to the U. N. Secretariat at the Plenipolentiary Conference convened to draft a protocol for the international control of psychotropic substances (E. L. May).

Malaria Chemotherapy

6-Bromo-9-(2-diheptylamino-1-hydroxyethyl) phenanthrene hydrochloride (I) synthesized in this laboratory in 1945 and found active in avian malaria, was resubmitted about three years ago for the Walter Reed program. This compound has proved almost 100% effective (life-saving in several cases) in resistant, often fatal Plasmodium falciparum malaria in field trials (troops) in Viet Nam and in hospital cases of soldiers returning from that area. In 49 of 50 cases, parasites were completely cleared in a single 6-day dosage regimen with no toxicity to the host. "Triple therapy" (quinine, dapsone and trimethoprim) the most effective combination used until now, gives at best 90% protection and with, at times, nearly unmanageable toxic effects. Compound I is completely effective against two unusually virulent strains of Plasmodium falciparum not handled by any other known drug or the "triple therapy" regimen (E. L. May).

SECTION ON METABOLITES

Histochemical Staining of Proteins: Mechanism of Diazo Coupling with Bound Tryptophan

One of the most widely used diazonium salts for histochemical diagnostic tests involves the use of "Fast Red-B." Until recently it was generally assumed that reaction occurs only with tyrosine or histidine. However, extensive studies with models have now led to a better understanding of the reaction of tryptophan derivatives with various diazonium salts and to the realization that tryptophan residues in proteins are also partners in this staining technique (Thomas F. Spande, LC, George Glenner, LP).

Synthesis of Potential Antiviral Epidisulfides

Diketopiperazines with disulfide bridges are the active part of naturally occurring antibiotics and antiviral agents, such as gliotoxin and sporidesmin. In spite of the synthesis of two model compounds by chemists of Charles Pfizer and by a group in Vienna, there are no comparable methods available for the introduction of sulfur functions into the α-position of tryptophan derivatives. This has now been possible for the first time by an adaptation of reactions developed by H. Leuchs (H. C. J. Ottenheym).

Inhibitors of Proline Hydroxylase

It is desirable to manipulate the activity of proline hydroxylase, which enters into the biosynthesis of collagen and plays a role in tissue regeneration, wound healing, and presumably in Marfan's syndrome, chondrosarcoma, acromegaly and other diseases. A group of biochemists at the Roche Institute of Molecular Biology under Sidney Udenfriend has previously found that the peptide hormone bradykinin is a good substrate of proline hydroxylase. It has now been found that substitution of the third amino acid, proline, of the initial sequence Arg-Pro-Pro-Gly... by 3,4-dehydro-L-proline leads to nonapeptides which inhibit proline hydroxylase. This approach to inhibitors of this interesting enzyme is being further pursued.

Synthesis of Potentially Antiviral and Carcinostatic Nucleotides

[α-P^{32}]-Cyclopropyluridine-5'-diphosphate and [5-^{14}C]-dihydrouridine-5'-diphosphate have been prepared by chemical methods. Both dihydrouridine-5'-diphosphate (H_2UDP) and cyclopropyluridine-5'-diphosphate (ΔUDP) are equally poor inhibitors of the enzyme polynucleotide phosphorylase. Neither H_2UDP nor ΔUDP is a substrate for polynucleotide phosphorylase (PNPase) when either compound is tested by itself using magnesium or manganese as metal cofactor. When $(A_p)_3A$ is used as a primer for the polymerization reaction, both H_2UDP and ΔUDP undergo polymerization, the maximum occurring at a one-to-one ratio of substrate to primer. Indications are that the products result from the addition of H_2UDP or ΔUDP to the primer. Both H_2UDP and ΔUDP can undergo copolymerization with UDP, CDP, IDP or ADP. The amount of H_2UDP or ΔUDP incorporated varies with the initial substrates ratio as well as with the metal ion, but in all cases H_2UDP is at least twice as effective as ΔUDP as a substrate in the copolymerization. The polymeric products from the copolymerization reaction have been deproteinized and purified by chromatography on Sephadex G-75. A copolymer consisting of U and [^{14}C]-H_2U is degraded by Pancreatic Ribonuclease to give products which indicate that the H_2U residues are internal in the polynucleotide chain as well as terminal. Preliminary indications are that the copolymer formed from ΔUDP and CDP or UDP is of the same nature. Purified samples of poly(C, H_2U) with C/H_2U = 100/5 and poly-(C, H_2U) with C/H_2U = 2/1 have been submitted to Dr. Sam Baron for testing as to their ability to induce interferon, either by themselves or annealed with Poly I (Paul Torrence).

Fluorescent Derivatives of Strophanthidin as Inhibitors of Sodium-Potassium-Dependent Adenosine Diphosphates

Strophanthidin derivatives, veratridine analogs and batrachotoxin A p-bromobenzoate and their parent compounds were tested as inhibitors of Na^+/K^+-dependent ATPase, measuring the rate of formation of ADP by recording the rate of NADH disappearance spectrophotometrically in the presence of excess phosphoenolpyruvate, pyruvokinase and lactic dehydrogenase. The strophanthidin 3-O-esters showed very strong Na^+/K^+-dependent ATPase inhibitory activities (appreciably stronger than ouabain) whereas the strophanthidin-19-hydrazones were less active. One of the most active inhibitors of this latter group, the strophanthidin 19-salicylhydrazone, showed strong fluorescence when irradiated with UV light. Other membrane depolarizing agents such as veratridine, batrachotoxin and related compounds showed no or very weak Na^+/K^+-dependent ATPase inhibition (J. A. Waters, F. Oesch).

Novel Pyrrole Esters of Alkaloids and Steroids

Since the powerful and selective ion transport agent batrachotoxin possesses a uniquely substituted pyrrole-3-carboxylic acid group, other esters of pharmacologically active steroids and alkaloids have now been prepared as potentially useful new tools for the neurophysiological laboratory. Esterification of veracevine, jervine and methyl reserpate with the labile pyrrole carboxylic acid has been achieved, which apparently involves a mixed trifluoroacetic-pyrrole carboxylic anhydride intermediate. Esterification of

other alkaloids and steroids by this method, i.e., scopoline, codeine, morphine, cholesterol and strophanthidin is now in progress. Conventional methods of esterification resulted in the decomposition of the pyrrole moiety (J. A. Waters).

Separation of Toxic and Neurophysiological Effects in Batrachotoxin, Veratridine and Related Steroidal Alkaloids

Batrachotoxin possesses a 3α,9α-oxide bridge. When this is opened reductively by sodium borohydride the new dihydrobatrachotoxin has lost most of its toxicity, but is still neurophysiologically active in nerve muscle preparations. Such an increase in the "therapeutic index" is highly desirable for studies involving the possible usefulness of batrachotoxin derivatives in disorders in which the mechanism of transport of ions such as sodium is impaired, e.g., in muscular dystrophy. A similar reductive cleavage of the 4,9-oxide bridge of veratridine, under carefully controlled conditions, has now furnished dihydroveratridine, which will be evaluated in the laboratory of Edson X. Albuquerque (J. A. Waters).

Alkali Sensitivity of 3-Methylpyrimidine Nucleotides

After the first and only successful isolation of 3-methyluridine from an enzymatic hydrolysate of soluble RNA, subsequent attempts to isolate 3-methyluridines from alkaline hydrolysates of yeast tRNA failed. We have now shown that 3-methyluridine is extremely labile to alkali and would not survive the conditions of standard basic hydrolysis (0.3 M alkali at 37° overnight) usually applied to RNA. In view of the importance of methylation in the process of viral infection the possible base lability of 3-methyluridine and other methylated pyrimidines and purines becomes a matter of interest and concern (Y. Kondo, J.-L. Fourrey).

Intramolecular Transfer of Energy in the Photocyclization of Pharmacodynamic Amines

The fluorescence of many electron-rich aromatics is quenched efficiently by methyl chloroacetate and chloroacetamide. The absorption spectra of the quenchers show no transition above 280 nm; thus no low-lying singlet states are available for classical excitation transfer. The absorption spectra of the aromatics in acetonitrile were unchanged by the addition of either methyl chloroacetate or chloroacetamide. The unquenched portion of the fluorescence has the same spectral distribution as that in solutions without quencher. It is of considerable interest that methoxy substitution, which presumably increases electron density of the aromatic nucleus, leads to more efficient quenching. Quenching rate constants are dependent upon solvent polarity. Absorption and emission properties of N-acetyltryptamine (I) and N-chloroacetyltryptamine (II) are essentially identical. However, the intensity of room temperature fluorescence of I is approximately 15 times that of II. Fluorescence from N-chloroacetyl-3,4-dimethoxyphenethylamine has also been found to be significantly less than that from N-acetyl-3,4-dimethoxyphenethylamine. This behavior is most certainly due to intramolecular singlet quenching by the chloro-substituted amide (O. Yonemitsu, Hokkaido University).

Synthesis and X-Ray Analysis of cis-3,4-Methylene-L-proline, the New Natural Amino Acid from Horse Chestnuts, and of its trans-Isomer

The addition of carbene by the cuprous chloride-catalyzed decomposition of diazomethane to 3,4-dehydro-L-proline led to a mixture of cis-3,4-methylene-L-proline (I) and trans-3,4-methylene-L-proline (II) in a ratio of 1:3.5. The cis- acid I was identical with the natural amino acid isolated from seeds of Aesculus parvifolia by a modified technique. Detailed X-ray analyses of the hydrochloride of I and of the free amino acid II gave complete bond distances, angles and computer stereograms. The bicyclic system approaches a boat conformation both in the cis- and in the trans-acid, a result which confirms the evaluation of the nmr data. The pyrrolidine ring of I and II has all four carbons in a plane, while the nitrogen atom and the cyclopropyl carbon are displaced to the same side of the plane. In this respect the conformation of the hetero ring differs significantly from all other natural pyrrolidine amino acids (Y. Fujimoto, F. Irreverre, I. L. Karle, U.S. Naval Research Laboratory).

Histrionicotoxin A and Dihydroisohistrionicotoxin A, the First Acetylenic Venoms Isolated from Animals

Our continuing studies of frog venoms have led us to the isolation of a unique active principle from the skins of the Colombian frog Dendrobates histrionicus. This compound (7-(cis-1-buten-3-ynyl)-8-hydroxy-2(cis-2-penten-4-ynyl)-1-azaspiro[5.5]undecane) contains a spiro-heterocyclic system unique in nature and is the first example of an acetylenic compound of animal origin. The structure of a dihydro-derivative of histrionicotoxin has also been determined: One of the -HC=CH-C≡CH groupings has been replaced by a $-CH_2-\overset{H}{C}=C=CH_2$ grouping. This is the first example of such an allenic group in a natural product of animal origin. Cholesterol and related sterols and three other alkaloids were also isolated from skin extracts of the same frog, Dendrobates histrionicus. The novel structure of histrionicotoxin, related as it is to that of other alkaloids active in cholinergic mechanisms, warrants a thorough study both of its chemistry and pharmacology (J. Daly, T. Tokuyama, Osaka University, I. L. Karle, U.S. Naval Research Laboratory, C. W. Myers, American Museum of Natural History).

Radio- and Photochemistry of Diterpene Precursors of Gibberelic Acid

During X-ray analysis of a natural diterpenoid isolated from a plant growing in the high Andes of Venezuela an interesting bond breakage was observed for the first time that would be of interest in connection with the biosynthesis of gibberelic acid. When such reactions were studied photochemically it was noticed that irradiation of methylkaur-15-en-19-oate (I) with a low-pressure ultraviolet lamp gave a crystalline oxirane derivative as the principal product. The structure of this compound is being elucidated by X-ray analysis. Ultraviolet irradiation of I with a high-pressure lamp under sensitizing conditions gave a dihydro derivative. Both reactions appear to be free radical in nature (J. Waters, I. L. Karle, U.S. Naval Research Laboratory, Alfredo Usubillaga, Universidad de los Andes, Merida, Venezuela).

New Approaches to α,β-Unsaturated Steroidal Ketones of Potential Cardiac and Neurophysiological Interest

Anodic oxidation of the steroidal glycidate derived from 5α-cholestan-3-one gave 3-acetyl-5α-cholest-2-ene (32%) and 3ξ-acetyl-3ξ-methoxy-5α-cholestane (11%). The cycloalkyl glycidates also gave α,β-unsaturated ketones and α-methoxy ketones. This method introduces a new synthesis of α,β-unsaturated ketones and α-methoxy ketones. These reactions assist the known synthetic routes to steroids of cardiac, endocrinological and neurophysiological importance (J. Waters).

SECTION ON MICROANALYTICAL SERVICES AND INSTRUMENTATION

During the year a total of 9 man years was expended in performing about 8000 chemical and 5000 instrumental analyses for the research staff at NIH and a limited number of other government scientists. About 30 elements and 5 or more functional groups are determined routinely, using ultra-micro, micro or semi-micro techniques as required. Special instrumentation and procedures are devised to meet unusual situations. The routinely available instrumental analyses include: UV, IR, NMR, and ORD spectroscopy as well as medium and high resolution mass spectrometry.

SECTION ON PHARMACODYNAMICS

Studies on Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

Catechol-O-methyltransferase, purified 400 fold from rat liver, consists of two enzymatically active glycoproteins with molecular weights in a ratio of approximately 1:2. This enzyme catalyzes the formation of two isomeric O-methylated products from substituted catechols. The proportion of these two products is dependent on the nature of the ring substituent, the pH and the concentration of divalent cation (Mg^{++}, Mn^{++}, Zn^{++}). The enzyme is inhibited by Co^{++}, Cd^{++}, Fe^{++} and Ni^{++}. Trace amounts of a tightly bound Mg^{++} fully activate the enzyme towards ionized catechols, while large amounts of Mg^{++} are needed for full activation towards unionized catechols. Three to four Mn^{++} are bound to the enzyme. The affinity of Mn^{++} for enzyme is increased by S-adenosyl methionine and unaffected by substrate. Kinetic analysis of interactions between enzyme, substrate, S-adenosylmethionine and metal ion activator provides information on the topography of the active site. The enzyme is activated by norepinephrine and other catecholamines suggesting that it is an allosteric protein whose activity can be controlled by the level of physiological substrates (C. R. Creveling).

3-Mercapto-analogs of dopamine, dopa and related compounds were synthesized and found to be potent irreversible inhibitors of catechol-O-methyltransferase (W. B. Lutz and C. R. Creveling).

Identification of amines in crude mixtures appears feasible by combination of dansylation and chemical ionization mass spectrometry (C. R. Creveling).

A simple radiometric assay for tryptophan hydroxylase has been developed using 5-tritiotryptophan prepared by reduction of 5-bromotryptophan with tritium gas of moderate specific activity, thereby avoiding radiolysis and resultant random labeling of the tryptophan ring system (J. Daly).

The 4-O-methyl derivative of dopa was synthesized (Kaubisch).

The Role of Cyclic Adenosine Monophosphate in the Central Nervous System

Formation of cyclic AMP from a compartmentalized precursor pool of adenine nucleotides labeled by incubation with adenine-^{14}C is regulated in a synergistic manner by separate "receptors" for adenosine, histamine, and either norepinephrine or serotonin. The nature of the receptor sites has been investigated, revealing guinea pig cerebral cortex as the first tissue to be reported in which stimulated formation of cyclic AMP is largely mediated by an "β-receptor" for norepinephrine. A variety of antidepressants such as amphetamine, ritalin, pipradol, α-ethyltryptamine, harmaline and desipramine stimulate cyclic AMP formation in cerebral cortical slices. Adenosine appears to stimulate cyclic AMP formation both by interaction with a regulatory "receptor" and b incorporation into, and thus enlargement of, the precursor pool. Depolarizing agents, various metabolic inhibitors and a number of adenosine analogs ultimately cause depletion of ATP in the precursor pool resulting in a blockade of cyclic AMP formation. Certain adenosine analogs such as 5-deoxyadenosine, however, appear to block cyclic AMP formation by direct inhibition of adenyl cyclase. Simultaneous measurement of the formation of cyclic AMP-^{14}C and of endogenous cyclic AMP revealed that the specific activity of cyclic AMP remains constant during stimulated formation in response to histamine, norepinephrine and ouabain (M. Huang, J. Schultz).

The Mechanism of Enzymatic Hydroxylation and the Role of Reactive Intermediates in Drug Metabolism

Microsomal epoxide hydrase consists of at least two homologous enzymes, a general epoxide hydrase and a "benzene oxide" hydrase. The activity of epoxides as substrates or inhibitors of the former enzyme is strongly dependent on the nature, number and stereochemistry of the epoxide ring substituents. Inhibition of styrene oxide hydration by such epoxides may be competitive as with phenyl 2,3-epoxypropyl ethers, noncompetitive as with cyclohexene oxide or uncompetitive as with the most potent inhibitor, 1,1,1-trichloropropylene-2,3-oxide. The enzyme is activated by alcohols and by metyrapone. *In vivo* inhibition or activation of epoxide hydrase has not been accomplished. Development of more sensitive radiometric assays has allowed detection of epoxide hydrase and aryl hydroxylase activity in adult but not fetal rat skin. Only hydrase activity was detected in skin tissue cultures (F. Oesch, N. Kaubisch, D. Jerina).

Methyl migration occurs during isomerization of 1,2-dimethylnaphthalene oxide to the corresponding 4-dialone providing another example of the NIH Shift during such isomerizations of arene oxides (N. Kaubisch).

cis-1,2-Dihydroxy-1,2-dihydronaphthalene was identified as the sole dihydrodiol product from naphthalene metabolism in a variety of bacteria,

thereby correcting earlier speculation on trans-dihydrodiol formation. A model hydroxylating system utilizing ferrous ions, O_2 and thiosalicylic acid forms both cis- and trans-1,2-dihydroxy-1,2-dihydronaphthalene (A. Jeffrey, D. Jerina).

Azide and disulfide add normally to benzene-3,6-^{2}H oxide, while methyl lithium adds 1,6 to form a cis-product with methyl and deuterium on the same ring carbon (A. Jeffrey, D. Jerina).

The NIH Shift occurs during formation of phenols by fungi (D. Jerina).

The low temperature photolysis of arene oxides leads to the unstable ketonic tautomers of phenols—the first synthesis of these compounds. These ketonic tautomers are of particular interest because they are intermediates in the NIH Shift and in the enzymatic formation of phenols. In addition, other remarkable photoproducts have been observed from the arene oxides.

SECTION ON STEROIDS

The Study of the Constituents of Solanum Xanthocarpum

A highly toxic component toward the housefly larvae is present in the extracts of S. xanthocarpum. Fractionation of the alcoholic extract yielded the following compounds: Solasonine, solamargine, solasodine, Δ^7-citrostanol-3-benzoate, Δ^7-citrostandiol(3β,22), Δ^7-citrostanol-3-O-glycoside, carpesterol, debenzoxycarpesterol, cycloartanol, cycloartenol, B-sitosterol, stigmasterol and campesterol. Although the three steroidal alkaloids have some larvaecidal activity, the most toxic substance is still unidentified (G. Kusano and Y. Sato).

The Synthesis of Solacongestidine and Solafloridine

Δ^5-Solacongestidine has been synthesized in the following manner: Kryptogenin diacetate is converted into the 16-thioketal derivative and desulfurized with Raney nickel. Alkaline saponification affords cholest-5-ene-3β,26-diol-22-one. Partial tosylation of the latter to the 26-tosyl derivative, iodination to the 26-iodo compound followed by amination under pressure give the deserved alkamine. The synthesis resembles somewhat the biogenetic pathway and further studies will be conducted with radioactive precursors (M. Nagai, Y. Sato and G. Kusano).

The Synthesis of Solaphyllidine

Attempted synthesis of solaphyllidine (22,26-epimino-5α-cholestan-4-one-3β,16α,23-triol 16-monoacetate) from Δ^5-solafloridine an alkamine present in S. congestiflorum is outlined as follows: Δ^5-solafloridine is transformed into the 3,16-diacetyl derivative, oxidized to the 23-oxo compound and reduced to the carbinol amine, 4-deoxo-Δ^5-solaphyllidine. The hydrogenated compound, 5,6-dihydro-4-deoxosolaphyllidine, was found to be identical with that derived from solaphyllidine. Introduction of the 4-oxo moiety into the molecule will be attempted (G. Kusano and Y. Sato).

The Study of Solanocapsine and its Derivatives

Solanocapsine hydriodide shows considerable activity in murine leprosy and the bisquaternary methiodide of solanocapsine exhibits neuromuscular blocking effect. Various derivatives of solanocapsine are being prepared as well as molecular modifications being made for testing the above mentioned activities (Y. Sato).

Steroidal Alkaloids - Reactions of Solasodine

A simple high yielding procedure for degrading solasodine to pregnadienolone in steroid hormone production is desirable. The treatment of 3-acetylsolasodine with phosgene in triethylamine followed by aq. dimethylamine affords 26-N',N'-dimethylcarbamido-4β-acetoxy-$\Delta^{5,22}$-furostadiene, N-(N',N'-dimethylaminoformyl) solasodine-3-acetate and 26-N',N'-dimethylcarbamido-3β-acetoxy-Δ^{5}-furosten-22-ol. The acetic acid treatment of the three compounds singly or combined yield 26-N',N'-dimethylcarbamido-3β-acetoxy-$\Delta^{5,20(22)}$-furostadiene which can be oxidatively degraded into pregnadienolone. This procedure may have some industrial significance (M. Nagai and Y. Sato).

Metabolism of C^{14}-Labelled Steroids by Adrenals from Pseudohermaphrodite Rats

A unique colony of rats is under study in which a genetic defect, carried by normal females, is passed on to half the genetic males. Half the genetic males are pseudohermaphrodites. These pseudos have both male and female characteristics, although the testes are internal. It has been demonstrated that these rats have a defect of testosterone synthesis in testicular tissue, suggesting a general deficiency of the 17-β-hydroxylase enzyme systems may be involved. This hypothesis is being investigated through in vitro studies with adrenal tissue and radioactive steroid precursors. Extracts of the incubation media were analyzed by both column chromatography and TLC. Significant quantitative differences in the metabolites of progesterone were found on comparison of pseudo adrenals with those from normal male and female. In addition to unchanged progesterone, 11-Keto-P,4-androstene-3,17-dione,11β-OH-androstenedione, the other metabolites were highest for the pseudo rat adrenal. Of particular interest was the higher production of 4-androstene-3,17-dione, a direct precursor of testosterone. These preliminary findings support the general hypothesis of 17β-hydroxylase deficiency. More definitive studies are continuing (D.F. Johnson and N. Lamontagne).

Column Chromatography of Adrenocortical Steroids and Ketosteroids by Gradient Elution

Chromatographic resolution of complex mixtures of adrenal steroids requires precise elution gradients that can be varied at will and maintained within precise limits of solvent concentration. Such a system has been developed in this laboratory which allows for mixing and pumping solvent mixtures to within ±0.2%. An integral part of the chromatographic process involved development of analytical parameters of column preparation, using water-impregnated silicic acid, and gradient design to effect separation of selected adrenal and ketosteroids of biological significance. Using the punched-tape gradient system, a standard method was developed for the analysis

of these two classes of steroids in a mixture on a single column. The method has been successfully applied to both model mixtures and those resulting from incubation experiments using adrenal tissue and radioactive steroid precursors (D. F. Johnson and N. Lamontagne).

Photodehydrohalogenations of Haloacyl Derivatives of Certain Amines and Hydroxy Compounds

N-Chloroacetyltryptophan photocyclizes in the 4-position of the indole moiety to form a tricyclic eight-membered lactam. N-chloroacetyl derivatives of tryptamine, 3-(3'-aminopropyl)indole and 3-(4'-aminobutyl)indole have been synthesized. Photolysis of these compounds in aqueous methanol with a mercury vapor lamp results in cyclization at the 4-position with the production in good yields of tricyclic eight-, nine-, and ten-membered lactams, respectively (C. M. Foltz, T. Kobayashi, and B. Witkop). In the course of this study a phototitration technique was developed whereby the course of the reaction is followed by automatic titration of the protons produced. This technique has been useful in the continuing study of the scope of the reaction and with further development has potential usefulness in the study of other photodehydrohalogenations and in the study of the application of photodehydrohalogenation reactions to the investigation of proteins and other macromolecules of biological interest (C. M. Foltz).

Phototitrations of substrates such as N-chloroacetyl derivatives of tryptamine, tryptophane, homotryptamine, 3-(4'-aminobutyl)indole, 5-hydroxytryptophane, 5-methoxytryptamine, 4-methyltryptophane, histamine, m-tyramine, O-methyltyramine and O-methyl-m-tyramine and N-bromoacetyltryptamine, B-chloropropionyltryptamine, β-chloropropionylhomotryptamine and γ-chlorobutyryltryptamine have shown that the rate of photodehydrohalogenation depends on the nature of the aromatic moiety, the nature of the haloacylamino group and the length of the group bearing the halocylamino function. Studies so far indicate that the rate of the reaction is indicative of the path of the reaction (C. M. Foltz).

The photocyclodehydrohalogenation reaction has been extended to aromatic polycarbocyclic systems with the photolysis of N-chloroacetyl-2-(α-naphthyl)-ethylamine. The reaction leads to a tricyclic lactam in yields comparable to those for lactam formation from N-chloroacetyltryptamine. The reactions in both series take place at comparable rates and rates and product composition are not changed significantly by conducting the reactions under oxygen instead of nitrogen. This suggests that free radical or triplet intermediates are not involved in the reactions (C. M. Foltz).

In the course of exploratory studies of the intermolecular photodehydrohalogenation reaction anomalous results were obtained when phenyl chloroacetate was one of the reactants. Phenyl chloroacetate, an ester derived from a phenol, undergoes the photo-Fries rearrangement as well as dehydrohalogenation. Various substrates have been prepared and some have been photolyzed in beginning a study of the photolysis of haloacid esters derived from aromatic compounds. This reaction may have applications of biological interest (C. M. Foltz).

The Structure Determination of N,N,N'-Trimethylsolanocapsine by X-ray Crystallography

The growth of *Mycobacterium leprae* and *M. tuberculosis* is suppressed by the *Solanum* alkaloid, solanocapsine. The N,N,N'-trimethyl derivative of the alkaloid gives monoclinic prisms suitable for x-ray diffraction study. In order to define the structure completely and in detail, plans have been made to collect x-ray diffraction intensity data with a crystal of the trimethyl derivative and to attempt a phase solution using direct methods (J. A. Beisler, Y. Sato and J. V. Silverton).

Serial No. NIAMD-LC-1
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Oxidation Mechanisms in Metabolic Processes

Previous Serial Number: SAME

Principal Investigator: Louis A. Cohen

Other Investigators: R. T. Borchardt and Y. Kikugawa

Cooperating Units: None

Man Years

Total: 1.3
Professional: 1.3
Other: 0

Project Description:

A study of the mechanisms by which energy derived from respiration is ultimately converted into labile bond energy, as in ATP; a study of the mechanisms by which molecular oxygen is activated for direct incorporation into substrate.

Objectives: To demonstrate that when oxygen is bound to metal-chelate systems, it is transformed from the triplet (diradical) state to the singlet (ionic) state and that the direct incorporation of oxygen into substrate involves the electrophilic attack of activated singlet oxygen. To study possible mechanisms for the conservation of oxidative energy by means of chemical models and to apply such knowledge to elucidation of the enzymatic pathways. To devise chemical models for the Mitchell hypothesis--that ATP formation can be coupled to a pH gradient and the free energy available therefrom.

Methods Employed: Infrared and ultraviolet spectroscopy are used to follow rates of reaction of intermediates and to determine structural characteristics. The latter method is also used for quantitative assay of labile materials. Nuclear magnetic resonance spectroscopy is used for structural elucidation of closely related materials. Various chromatographic procedures, particularly on thin layer plates, are used extensively.

Major Findings: In an extension of earlier work on the conservation of oxidative energy in chemical bonds, a conformationally restricted analog

of ubihydroquinone has been oxidized in acetic acid solution to generate a mixed acid anhydride. In the presence of ADP and inorganic phosphate, the free energy of the anhydride linkage is utilized to generate ATP and to release the corresponding quinone. Reduction of the quinone spontaneously regenerates the starting compound, ready for another cycle. By a repetitive cycle of oxidative and reductive steps, the ubiquinone analog may be used as a catalytic cofactor, converting ADP into ATP continuously by consumption of an oxidant.

A model has been designed to test the Mitchell hypothesis, by applying the principle of stereopopulation control to an imidazole derivative. A series of obstacles in the synthesis of the model has been overcome and kinetic studies are being undertaken.

Significance to Bio-medical Research and the Program of the Institute: The mechanism by which molecular oxygen is carried into the cell, bound to hemoglobin, has been well elucidated. Its subsequent fate remains obscure. One immediate goal of this project is to determine the mechanisms by which oxygen is activated to a state in which it can attack a variety of relatively inert substrates.

Similarly, the function of ubiquinone in oxidative phosphorylation has yet to be elucidated. It has been shown that oxidative energy is utilized in the conversion of ADP to ATP in mitochondria, ubiquinone being an essential cofactor.

The present findings permit a novel role to be assigned to ubiquinone, Vitamin K and other quinone cofactors.

Controversy regarding the Mitchell hypothesis for mitochondrial synthesis of ATP has continued unabated, since no experiment has been devised to test its validity. The model system under study will be used to attempt to settle the question of whether ATP can be synthesized simply by a rapid change in pH.

Proposed Course: Studies will be continued on bond conservation of oxidative processes and efforts will be made to bridge the gap between the chemical model and the mitochondrion.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-2
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Chemical Modification and Cleavage of Proteins

Previous Serial Number: SAME

Principal Investigator: Louis A. Cohen

Other Investigators: S. Takahashi and Kenneth L. Kirk

Cooperating Units: None

Man Years

Total: 0
Professional: 0
Other: 0

This project has been temporarily discontinued.

Serial No. NIAMD-LC-3
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Cleavage of Peptide Bonds by Intramolecular Participation and Mechanisms of Action of Hydrolytic Enzymes

Previous Serial Number: SAME

Principal Investigator: Louis A. Cohen

Other Investigators: Kenneth L. Kirk and Yasuo Kikugawa

Cooperating Units: None

Man Years

Total: 0
Professional: 0
Other: 0

This project has been temporarily discontinued.

Serial No. NIAMD-LC-4
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Fluoro Analogs of Enzyme Substrates

Previous Serial No.: NONE

Principal Investigator: Louis A. Cohen

Other Investigators: Kenneth L. Kirk

Cooperating Units: None

Man Years

Total: 1.0
Professional: 1.0
Other: 0

Project Description:

Development of new methods for the synthesis of fluoro analogs of biochemically significant compounds and examination of their properties as enzyme inhibitors and as potential drugs.

Objectives: Fluoro analogs of steroids, purines, pyrimidines and amino acids have been synthesized and tested, several having been found valuable in arthritis and cancer chemotherapy. A larger number have not been prepared because of the limited routes available for the introduction of fluorine. Despite the ubiquitous role of imidazoles in biological systems (RNA, DNA, histidine, histamine, etc.), no fluorinated imidazole has yet been prepared. The initial purpose of this work was to develop a general method suitable for the preparation of fluoroimidazoles, to prepare a series of such compounds, and to test their abilities to serve as enzyme substrates or inhibitors and as replacements for histidine in proteins or for purines in DNA and RNA.

Methods Employed: Ultraviolet irradiation is used as the key step in the introduction of fluorine. Infrared, ultraviolet, mass, and nmr spectroscopy are used to follow the course of reactions and to elucidate product structure. Various chromatographic procedures are used for analysis and purification of materials.

Major Findings: Ultraviolet irradiation of imidazolediazonium fluoborates, in fluoboric acid solution, leads directly and in good yield to

the desired fluoroimidazoles. To date, this novel method of synthesis has provided 2-fluoroimidazole, 4-fluoroimidazole, 4-fluoroimidazole-5-carboxamide, and 4-fluoro-DL-histidine. The amino acid analog is deaminated slowly by histidine deaminase, while being bound to the enzyme much more tightly than the natural amino acid. This analog has also been found to have a soporific or hypnotic action on mice.

Significance to Bio-medical Research and the Program of the Institute: Past experience with fluoro analogs (fluorocitrate, fluorocortisone, and fluorouracil) warrants a search for other medicinally useful fluoroanalogs. Recent findings on the value of histidine in arthritis therapy, of imidazolecarboxamide in skin cancer therapy, and of histamine as a false neurotransmitter emphasize the magnitude of directions in which fluoroimidazoles may be examined clinically.

Proposed Course: Fluoroimidazoles already prepared will be submitted for pharmacological testing and will be examined as enzyme substrate analogs. Additional and more complex compounds will be prepared for the same purpose. The photochemical method of synthesis will be applied to other types of heterocyclic systems, in addition to imidazole. Fluorine nuclear magnetic resonance spectroscopy will be used to study enzyme-substrate complexes with a view to aiding in the elucidation of mechanism of enzymes such as histidine deaminase.

Honors and Awards: Invitation to report on photochemical fluorination at an American Chemical Society Symposium on Fluorine and Medicinal Chemistry, Washington, D.C., September, 1971.

Publications:

Kirk, K. L. and Cohen, L. A.: Photochemical decomposition of diazonium fluoborates. Application to the synthesis of ring-fluorinated imidazoles. J. Amer. Chem. Soc., in press.

Serial No. NIAMD-LC-5
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: General Principles of Enzyme Catalysis and Simulation

Previous Serial Number: NONE

Principal Investigator: Louis A. Cohen

Other Investigators: S. Milstien and R. T. Borchardt

Cooperating Units: None

Man Years

Total: 2
Professional: 2
Other: 0

Project Description:

A study of the principles used by enzymes to accelerate chemical reactions. Model compounds are designed and synthesized which are capable of undergoing various reactions at rates comparable to those of enzymes, and under the same reaction conditions. Recognition of the principal devices used by enzymes will permit the design of more effective drugs and enzyme inhibitors or substrates.

Objectives: The rates of enzyme-catalyzed reactions exceed those of simple test-tube analogs by factors of 10^{10}-10^{18}. In order to account for such a phenomenal effect, model compounds which duplicate one or more of an enzyme's special powers are studied to learn more about the vastly complex protein catalyst. In the belief that the principal enzymatic device is stereopopulation control (near perfect orientation of functional groups), model compounds have been designed and synthesized in which rotation of covalent bonds has been severely restricted. By bringing two functional groups into very close proximity and orientation, rates of reaction comparable to those of enzymes can be achieved in the test tube.

Methods Employed: Spectroscopic methods are used to elucidate the structures of synthetic products and to follow rates of reaction. Various chromatographic procedures are employed for analysis and purification.

Major Findings: Stereopopulation control has been applied to the design of a model for nucleophilic displacement, using a phosphate as leaving group

and a phenol as nucleophile. The freezing of rotation of single bonds increases reactivity to such a point that the half-life of the most reactive compound, at 30° and pH 8, is 0.2 sec. The rate of this reaction exceeds that of phenol with triethyl phosphate by a factor of 10^{11}.

Similarly, a model system has been designed to illustrate ester formation. By application of the new principle of stereopopulation control, the reaction can be made to occur with a half-life, at pH 6 and 30°, of 0.2 sec. The rate of ester formation in the model system exceeds that of phenol with acetic acid by a factor of 10^{15}.

Significance to Bio-medical Research and the Program of the Institute: Despite the fast amount of research invested in the elucidation of the pathways of specific enzyme-catalyzed reactions, justifications of the rates of these reactions, in comparison with their nonenzymatic counterparts, are still in a primitive state. A sound understanding of the means by which enzymes operate so effectively would lead not only to a better appreciation of biochemical processes in general, but also to a more rational approach to drug design.

Proposed Course: Utilizing the principles of stereopopulation control already developed, additional models will be studied to produce even faster reaction rates than those already observed. The principle will also be extended to other types of reactions, such as hydride transfer and addition/elimination. The control principle will also be used to design conformationally restricted analogs of various drugs with a view to enhancement of activity and reduction of side-effects.

Honors: Invitation to report on the new concept of stereopopulation control at a Symposium on Enzyme Mechanisms, Santa Barbara, California, December 1970, and at the Institute for Cancer Research, Philadelphia, February 17, 1971.

Publications:

Milstien, S. and Cohen, L. A.: Rate acceleration by stereopopulation control: models for enzyme action. Proc. Nat. Acad. Sci. U.S. 67: 1143-1147, 1970.

Serial No. NIAMD-LC-6
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Higher-Carbon Sugars and Their Derivatives.

Previous Serial Number: same

Principal Investigator: N. K. Richtmyer

Other Investigators: None

Man Years

Total: 1
Professional: 1
Other: None

Project Description:

Objective: To evolve generalizations relating the physical and chemical properties of the groups of substances named in the project title to their configurations and conformations.

Methods Employed in Major Findings: The isolation of a talo-heptulose (presumably the D form) from the avocado was reported from this laboratory in 1960. The isolation of D-allo-heptulose from Primula Officinalis roots was reported from this laboratory in 1966. Recently, reinvestigation of the syrupy talo-heptulose sample, by both gas-liquid chromatography and paper chromatography, showed it to contain both a talo-heptulose and an allo-heptulose.

Syrupy D-threo-L-ido-octose was found to be converted, by long standing, into D-glycero-L-gluco-octulose. The same crystalline octulose was also obtained by oxidation of D-threo-L-gulo-octitol with Acetobacter suboxydans. Treatment of D-glycero-L-gluco-octulose with methanol and an acid catalyst produced crystalline methyl D-glycero-α-L-gluco-octulopyranoside, whose structure was proved by degradation to methyl α-L-gluco-heptulopyranoside; the last-named compound is the evantimorph of methyl α-D-gluco-heptulopyranoside, whose pyranoside structure was proved in 1942.

Significance to Biomedical Research and the Program of the Institute: As mentioned before, sedoheptulose and D-manno-heptulose (both of which occur in various families of plants) have

been found to be of significance in metabolism (both animal and plant) and in studies on diabetes, respectively. The significance of the presence of other heptuloses, as well as of octuloses, and nonuloses in plants is still unknown.

Proposed Course: In the few months remaining before my compulsory retirement, I propose to study the octulose and the nonuloses that have been prepared by the oxidation of an octitol and two nonitols, respectively.

Honors and awards: None

Publications:

Johansson, I., and Richtmyer, N. K.: The isolation of both a talo-heptulose and an allo-heptulose from the avocado. Some new paper-chromatographic data on heptuloses. Carbohyd. Res. 13: 461-464 (1970).

Richtmyer, N. K.: Crystalline D-glycero-L-gluco-octulose, crystalline methyl D-glycero-α-L-gluco-octulopyranoside, and some related compounds. Carbohyd. Res. 17:401-410 (1971).

Serial No. NIAMD-LC-7
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunochemistry and Reactions of Carbohydrates.

Previous Serial Number: 5

Principal Investigator: C. P. J. Glaudemans

Other Investigators: M. Bertolini

Cooperating Units: None

Man Years

Total: 2
Professional: 2
Other: None

Subtitle: Investigation of four types of D. pneumoniae related by an apparent mutation.

Project Description:

Objective: D. pneumoccus type 61 cross reacts with serum to type 31, and will mutate to type M61 in its presence. Type M61 then cross reacts with Type 13, and M61 will revert to the parent 61 if subcultured serially in the presence of serum to type 13 (Laros, 1949). We are investigating this relationship, and first of all, are elucidating the structures of the type specific capsular polysaccharides occurring on the organisms which are involved.

Methods Employed: All modern and classical techniques are called upon for kinetic and inhibition work, and in the elucidation of the structure of antigens and the synthesis of substrates. These involve hydrolysis studies, methylation, glc, mass spectrometry, n.m.r. and synthesis.

Major Findings: The major work done on this project was the preparation of pure capsular antigen. The species specific C-antigen in all our preparations of Type 61 capsular polysaccharide could be removed by precipitation with TEPC-15 Myeloma protein (from Dr. M. Potter). The antigen is then isolated by chromatography over DEAE cellulose columns.

Subtitle: The oxidation of glycosides by chromium trioxide.

Objective: Angyal et al. have reported the oxidation of methylglycosides by chromium trioxide-acetic acid. Using benzyl glycosides, we have used the procedure to prepare 1-O-benzoyl glycosides (see last year's report) and we have shown that the manner of oxidation depends very much on the nature of the aglycon.

Major Findings: We are presently investigating the oxidation of gentiobiose and isomaltose β-octa acetates. It has been found that for methyl glycosides the β anomer is oxidized much more rapidly than the α-anomer. If this is so for disaccharides, it may be possible to oxidize β1→6 linkages selectively. We have oxidized model compounds such as methyl α-D-glucopyranosides tetra-acetate to methyl-5 keto gluconate. The latter compound can be reduced by sodium borohydride in CO_2 buffered isopropanol to a mixture of L-iditol and D-glucitol. The oxidation of an acetylated 1→6 disaccharide can give either (or both) of two products: 6-O-(tetra acetyl 5 keto gluconyl) glucose tetra-acetate (A) or tetra-acetyl glucosyl-glucuronate tetra-acetate (B). Upon treatment with sodium borohydride in CO_2 buffered isopropanol A should yield glucose, iditol and glucitol, whereas B should yield glucose only.

Subtitle: Synthesis of indolyl-α-D-galactopyranosides.

Objective: Fabry's disease is one of the sphingo lipidoses. Its diagnosis at present involves a rather complicated procedure. It would be desirable to have available a facile diagnostic text, such as is the case in Gaucher's disease. To this end, indolyl-α-D-galactoside would be excellent. In the case of normal tissue, α-galactosidase would hydrolyticall cleave the indolyl group causing the formation of indigo in the tissue. This is now the test for Gaucher's disease (using indoly-β-D-glucoside).

Major Findings: The synthesis of α-D-galactoside - glycosides having a 1,2 cis arrangement - poses difficulties. The halide has to be used and if the usual approach is employed reacting α-acetobromo galactose, the resulting glycoside invariably appears to be the β-anomer. There is one exception, namely α-phenyl glycosides of galactose have been prepared from acetobromo galactose using quinoline as a catalyst. We are presently investigating this for our purpose.

Another possibility is the use of a galactosyl halide masked by benzyl groups, since these do not participate in the reaction at C-1. We have prepared tetra-O-benzyl galactosyl chloride in high yield. We are presently investigating its reactions with N-acetyl indoxyls.

Significance to Biomedical Research and the Program of the Institute: Work on the immuno determinant groupings in biopolymers will contribute to our general knowledge of immunity. The study of four related antigens appears to be a good vehicle to clarify the reasons for different immunochemical behavior due to minor variations in structure. The work on enzyme substrates such as indolyl-galactosides will, if successful, lead to an excellent diagnostic test for an important disease.

Honors and awards: None

Publications:

Maurice Bertolini and C. P. J. Glaudemans: The chloroacetyl group in synthetic organic chemistry, Carbohyd. Res. 15:263 (1970).

C. P. J. Glaudemans: The carbohydrates, chemistry and biochemistry. Vol. II B. Edited by W. Pigman and D. Horton. Book review. Carbohyd. Res. 17:252 (1971).

Maurice Bertolini and C. P. J. Glaudemans: Oxidation of alkyl glycosides, Carbohyd. Res. 17:449 (1971).

Maurice Bertolini and C. P. J. Glaudemans: 1,2,5-Ethylidyne-α-D-galactose, Carbohyd. Res. 18:131 (1971).

C. P. J. Glaudemans and H. G. Fletcher: The mechanism of formation of some pentofuranosyl halides. J. Org. Chem. In press.

Serial No. NIAMD-LC-8
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Synthesis of Carbohydrate Derivatives for Biomedical Research

Previous Serial Number: 4

Principal Investigator: H. G. Fletcher, Jr.

Other Investigators: H. W. Diehl, R. K. Ness, E. W. Tracy, E. Zissis.

Cooperating Units: Dr. N. Pravdic, Department of Organic Chemistry, "Ruder Boskovic" Institute, Zagreb, Yugoslavia (under P.L. 480).

Man Years

Total: 7.3
Professional: 4.3
Others: 3

Part A. The Oxidation of 2-Acetamido-2-deoxyaldoses

Project Description:

Objective: Experiments with highly impure substrates have shown that 2-acetamido-2-deoxy-D-glucono-1,5-lactone is a remarkably effective competitive inhibitor of epididymal β-N-acetylglucosaminidase. The objective of the present project is to develop methods for the synthesis of this lactone and to extend our knowledge of compounds in this class.

Major Findings: 2-acetamido-2-deoxy-D-galactose and 2-acetamido-2-deoxy-D-mannose are readily oxidized by unbuffered aqueous bromine to give corresponding 2-acetamido-2-deoxy-D-aldono-1,4-lactones in crystalline form and in high yield. While 2-acetamido-2-deoxy-D-glucose is also readily oxidized by aqueous bromine, it yields a mixture which includes 2-amino-2-deoxy-D-gluconic acid, 2-acetamido-2-deoxy-D-gluconic acid, 2-acetamido-2-deoxy-D-glucono-1,5-lactone and at least one other, probably isomeric, lactone. 2-Acetamido-2-deoxy-D-gluconic acid may be isolated from the mixture as its salt with dicyclohexylamine; from the mother liquor 2-acetamido-2-deoxy-D-mannono-1,4-lactone may then be isolated and it seems

likely that the isomerization involved here is due to the substantial alkalinity of dicyclohexylamine. When treated with methanolic potassium hydroxide, the two 2-acetamido-2-deoxy-D-aldono-1,4-lactones (of the D-galactose and D-mannose series), as well as the reaction mixture from the oxidation of 2-acetamido-2-deoxy-D-glucose, give the same mixture of two substances. These were shown to be the 2-acetamido-2,3-dideoxyhex-2-enono-1,4-lactones of the D-threo and D-erythro series. Their formation is reminiscent of the elimination shown by O-glycosides of serine and threonine moieties in glycoproteins but appears to take place with even greater ease.

A closely allied endeavor has concerned the oxidation of 2-acetamido-2-deoxyhexopyranose derivatives that are substituted at all positions except carbon 1. Thus 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-D-glucopyranose was found to be oxidized by dimethylsulfoxide-acetic anhydride to 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-D-glucono-1,5-lactone in nearly quantitative yield. Hydrogenolytic removal of the benzyl groups from this lactone gave 2-acetamido-2-deoxy-D-glucono-1,5-lactone which was isolated in pure crystalline form; this is the first unequivocal synthesis of this substance. On treatment with dimethylamine, 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-D-glucono-1,5-lactone gives 2-acetamido-3,4,6-tri-O-benzyl-2-deoxy-di-N-methyl-D-gluconamide which is then partially epimerized by the action of the amine to the corresponding D-mannoamide derivative. Similarly, the crude reaction mixture from the bromine-water oxidation of 2-acetamido-2-deoxy-D-glucose gives 2-acetamido-2-deoxy-di-N-methyl-D-mannonamide when treated with dimethylamine.

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-D-glucose and -D-mannose are oxidized with dimethylsulfoxide-acetic anhydride to the corresponding 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-D-hexono-1,5-lactones but each is accompanied by 2-acetamido-4,6-di-O-acetyl-2,3-dideoxy-D-erythro-hex-2-enono-1,5-lactone. It is thus evident that β eliminations of this type can take place in six-membered as well as in five-membered lactone ring structures.

Significance to Biomedical Research and the Program of the Institute: A new and practicable synthetic route to an interesting enzyme inhibitor has been developed and some knowledge of the chemistry of 2-acetamido-2-deoxyaldonic acids has been obtained.

Part B. Unsaturated Aminosugars

Project Description:

Objective: Earlier work led to the discovery of methods for the synthesis of 2-acetamido-2-deoxyhexose derivatives having a double bond between carbon atoms one and two. One of these substances, 2-acetamido-D-glucal was incidentally found to be a moderately effective inhibitor of β-N-acetylglucosaminidase. The immediate objective of the present project was to explore some of

the chemical properties of this new class of unsaturated sugars with their potential utility for synthetic purposes in mind.

Major Findings: Under fusion conditions and in the presence of a trace of p-toluenesulfonic acid, various acids and phenols have been found to attack 3,4,6-tri-O-acetyl-2-(N-acetylacetamido)-1,2-dideoxy-D-arabino-hex-1-enopyranose. The acetyl grup at C-3 is lost, the double bond shifts to the C-2-C-3 position and the attacking species enters at C-1; this, at least is the superficial view of the overall reaction. Actually, closer study shows that the unsaturated aminosugar derivative initially undergoes an allylic rearrangement, the acetoxy group at C-3 migrating to C-1; there it is subsequently displaced by the attacking nucleophile.

Significance to Biomedical Research and the Program of the Institute: A synthetic pathway to the 2-acetamido-2,3-dideoxyaldohexoses has been opened up; derivatives of this new class of aminosugar may have interesting biochemical properties.

Part C. Synthesis with Partially Benzylated Sugars

Project Description:

Objective: The synthesis of 1,3,4,6-tetra-O-benzyl-D-fructofuranose was described in an earlier report. During the past year some chemical properties of this and other benzyl ethers of the carbohydrates were investigated with a view to future synthetic efforts.

Major Findings: 1,3,4,6-Tetra-O-benzyl-D-fructofuranosyl chloride is readily preparable from 1,3,4,6-tetra-O-benzyl-D-fructofuranose. However, the substance appears to be of limited synthetic utility owing to the ease with which it undergoes dehydrohalogenation. This reaction produces two unsaturated compounds that were shown to be 1,3,4,6-tetra-O-benzyl-2-deoxy-D-erythrohex-2-enulofuranose and 1,3,4,6-tetra-O-benzyl-2-deoxy-D-arabino-hex-1-enulofuranose.

The chemical synthesis of sucrose has presented a baffling and challenging problem for many decades and, prior to the period under report, has only once been carried out successfully. We have found that the condensation of 1,3,4,6-tetra-O-benzyl-D-fructofuranose with 2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl chloride affords octa-O-benzylsucrose from which the benzyl groups were readily removed through catalytic hydrolysis. The octabenzyl ether of α-D-glucopyranosyl α-D-fructofuranoside is also formed in the reaction and was found to be a product of the condensation of 1,3,4,6-tetra-O-benzyl-D-fructofuranose with 2,3,4,6-tetra-O-benzyl-D-glucopyranose in the presence of perchloric acid.

A highly novel dibenzylation procedure has been discovered.

With 1,2-ethanedithiol and boron trifluoride etherate at room temperature, 2,3,4,6-tetra-O-benzyl-D-glucopyranose gives D-glucose ethylene dithioacetal, the benzyl groups appearing as benzyl sulfide and tribenzylsulfonium tetrafluoroborate. Monovalent thiols may also be used. Thus ethanethiol and boron trifluoride etherate with the above substrate gives a mixture of the anomeric ethyl 1-thio-D-glucopyranosides and dibenzyl ethyl sulfonium tetrafluoroborate. This debenzylation procedure has been found well-suited to the identification of the anti-inflammatory drug "Glyvenol."

Honors and awards: None

Publications:

Pravdic, N., Zidovec, B. and Fletcher, H. G., Jr.: Allylic Displacement Reaction of a 2-Acetamido-D-glucal Derivative with Acids and Phenols. Croat. Chem. Acta 42:523-533, 1970.

Zissis, E., Ness, R. K. and Fletcher, H. G., Jr.: Two Benzylated Hydroxyglycals derived from D-Fructofuranose. Carbohyd. Res.In press.

Fletcher, H. G., Jr. and Diehl, H. W.: De-O-benzylation of Carbohydrate Derivatives by Thiols in the Presence of Boron Trifluoride. Carbohyd. Res. In press.

Ness, R. K. and Fletcher, H. G., Jr: Synthesis of Sucrose and of α-D-Glucopyranosyl α-D-Fructofuranoside Through the Use of 1,3,4,6-Tetra-O-benzyl-D-fructofuranose. Carbohyd. Res. In press.

NIAMD-LC-9
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: 1) The photocyclization of cis 9-chloroacetamido-5-(3-hydroxyphenyl)-2-methyl-2-azabicyclo[3,3,1]nonane.
2) The syntheses and pharmacological evaluation of 9-hydroxy-4,10b-iminoethano-11-methyl-1,2,3,4,4a,5,6,10b-octahydrobenz[c]quinolines.
3) The syntheses and pharmacological evaluation of 10-hydroxy-4,11b-iminoethano-12-methyl-1,2,3,4,4a,6,7,11b-octahydro-5H-dibenz[b,d]azepines and their 8-hydroxy analogs.

Previous Serial Number: Same

Principal Investigators: Helen H. Ong

Other Investigators: Everette L. May

Cooperating Units: None

Man Years:
Total: 1
Professional: 1
Other: 0

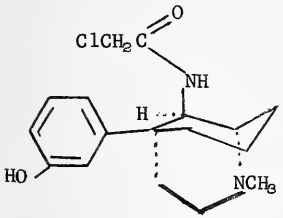
Project Description:

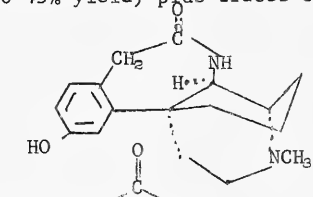
Objectives: 1) To further explore the synthetic applicability of the intramolecular photocyclizations discovered by B. Witkop, et.al., in complex systems with fixed steric configurations. 2) To synthesize and evaluate new classes of analgesics on morphine antagonists which may be of therapeutic value. 3) To study the structure-activity relationship of these compounds.

Methods Employed: Synthetic techniques, tlc, vpc, uv, ir, mass and nmr spectroscopy.

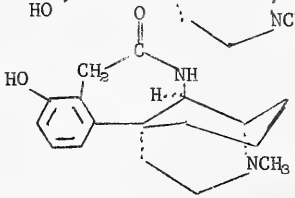
Major Findings: 1) 5-(3-Methoxyphenyl)-2-methyl-9-oxo-2-azabicyclo-[3,3,1]nonane was converted via its oxime to cis-9-amino-5-(3-methoxyphenyl)-2-methyl-2-azabicyclo[3,3,1]nonane in which the amine group is axial. The trans isomer could be obtained in 70% stereospecificity when Na/C_2H_5OH was used as the reducing agent. The former (cis compound) was O-demethylated and chloroacetylated in $CHCl_3$. Brief sapnification of the bis-chloroacetylated compound followed by acidification gave cis-9-chloroacetamido-5-(3-hydroxyphenyl) 2-methyl-2-azabicyclo[3,3,1]nonane (I) (90%). 2) Photolysis of I

with a high pressure Hg. Lamp (Vycor) yielded two compounds (II and III) in approximately equal concentrations (each in 40-45% yield) plus traces of





II



III

other products. The total as well as relative yields of II and III seemed to be independent of the pH of the photolylic solution. II and III were both shown to be lactams (by ir, uv, mass elementary analyses) resulting from ring closures with simultaneous release of HCl. Structures II and III were tentatively assigned on the basis of nmr; the splitting pattern and coupling constants of the three aromatic protons in II are such that two H's are ortho to each other while the third one is isolated and metacoupled to one of the adjacent pairs.

2) II and III were separated by fractional recrystallization and were converted, separately, to their methoxy derivatives by CH_2N_2. Reduction with diborane followed by O-demethylation with HBr yielded 10-hydroxy-4,11b-iminoethano-12-methyl-1,2,3,4,4a,6,7,11b-octahydro-5H-dibenz[b,d]azepine and the 8-hydroxy analog, respectively. Their 5-methyl homologs were synthesized by reductive methylation with HCHO/HCOOH prior to O-demethylation.

3) Cis-9-amino-5-(3-methoxyphenyl)-2-methyl-2-azabicyclo[3,3,1]nonane underwent Pictet Spengler reaction to give 9-methoxy-4,10b-iminoethano-1,2,3,4,4a,5,6,10b-octahydrobenz[c]quinoline, which was then demethylated to the 9-hydroxy compound. Its 5-methyl (N-methyl) homolog was synthesized similarly by reductive methylation of the methoxyl compound prior to O-demethylation.

Significance: 1) Photocyclization of chloroacetyl compounds proves to be useful in synthesizing complex ring systems which may otherwise be difficult to obtain.

2) Ortho ring closure was observed along with para-; previously, only para-ring closure was observed in systems incorporating monofunctional phenols.

3) The end products described above bear close structural resemblance to 5-(m-hydroxyphenyl)-2-methyl-morphan as well as to some new, potent analgesics in which a nitrogen atom is present in ring B. Preliminary pharmacological results indicate that some of these compounds possess

significant analgetic properties while others may be effective as morphine antagonists.

Proposed Course of the Project: 1) To seek more chemical proof for II and III. Preliminary results showed that III underwent acid rearrangment to give a diamine-γ-lactone (ir: 1800 cm^{-1}).

2) To attempt optical resolution of the racemic compounds found to have significant analgetic activity; and to compare the optical antipodes in analgesic action, physical dependence capacity and antagonistic behavior.

3) To synthesize compounds related to asparagine and/or asparaginyl peptides as possible anti-tumor agents.

Publications: Ong, H. H. and May, E. L.: Photocyclizations. I. 4,5-Dihydro-1H-Naphth[1,8-de]azocin-2[3H]-One. J. Org. Chem., 35, 2544, 1970.

NIAMD-LC-10
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: 1) Molecular modification of 9-oxo-9-10-dihydroanthracene
2) Derivatives of D-aspartic acid as potential antitumor agents.

Previous Serial Number: same

Principal Investigator: J. Harrison Ager

Other Investigators: Everette L. May

Cooperating Units: Pharmacology Unit of this Section

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: 1) To continue the study of the Mannich and other alkylation reactions of 9-oxo-9,10-dihydroanthracene as a springboard to physiologically active compounds and for theoretical reasons. 2) Make derivatives of D-aspartic acid to screen as potential antitumor agents.

Methods Employed: Standard methods of organic, physical-organic, and analytical chemistry.

Major Findimgs: When 9-oxo-9,10-dihydroanthracene was subjected to the Mannich Reaction which consists in the condensation of a primary or secondary amine, usually as the hydrochloride, with formaldehyde or paraformaldehyde and a compound containing at least one hydrogen atom of pronounced reactivity, the normal Mannich product was not obtained. The essential feature of the reaction is the replacement of the active hydrogen by an aminomethyl or substituted aminomethyl atom. This was not the case. The C,H, and N analysis of the product obtained showed a higher percentage of carbon and hydrogen than expected with no nitrogen. Mass sprectral data showed a molecular weight of 208. This coupled with a repeat of the C, H analysis, IR and nmr revealed the product to be methylated at the 10 position. Changing the reaction conditions - increased temperature, 0.25 cc hydrochloric

acid dissolved in 95% ethanol afforded the identical product gotten previously. Employing DMF at elevated temperatures afforded a product that at present has not been identified. Attempts were made to alkylate the ketone using sodamide in benzene to make the sodium derivative then adding dimethyl aminoethyl chloride and refluxing for 4-6 hr. An unidentifiable oil was obtained.

Derivatives of D-aspartic acid were made starting with N-carbobenzoxy-D-aspartic acid and using two equivalents of either phenethylamine or piperidine and diisopropyl carbodiimide to form the diamide. The bispiperdide derivative obtained was treated with hydrogen (catalytic) in the presence of 5% Pd-$BaSO_4$ to afford the amino diamide. This was treated with LAH (refluxing) to afford the amino derivative isolated as a crystalline trihydrochloride. The N-methyl compound of the carbamate was obtained by reducing it with LAH. The N-dimethyl compound was prepared from the N-methyl derivative by treatment with formaldehyde and formic acid and isolating the product as the trihydrobromide.

Significance: This basic and applied research falls within the frame work of this Institute and makes available new compounds of pharmacologic interest.

Proposed Course of the Project: To study the Mannich and base-catalyzed alkylation reactions on 9-oxo-9,10-dihydroanthracene under varying conditions. If successful will perform other operations on the products and examine them for biological activity. Additional modification of the D-aspartic acid molecule will be made again with a view toward antitumor agents. Work is planned on the substituted carbocyclic acids to form oxazines and oxadiazine.

Publications: None

NIAMD-LC-11
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: 1) Antimetabolites
2) Instrumentation

Previous Serial Number: Same

Principal Investigators: T. D. Perrine

Other Investigators: None

Cooperating Units: Ira B. Tice (NIH, R-BEI)

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: 1) To develop the synthesis of polymers carrying metabolites, primarly for anti-leukemia studies. 2) To develop new and improved methods of instrumentation.

Major Findings: 1) As would be expected, the anti-leukemia polymers are ineffective when given by routes in which the polymers are barred from reaching the target organ. After some delay, these materials are being tested in blood stream leukemia.

Chemical studies aimed at developing better polymeric carriers are in progress. These involve the synthesis of vinyl monomers with amino or phenolic functions which can serve to covalently link the haptene or metabolite in question. After purification these monomers can be suitably co-polymerized. Present efforts are directed to the preparation of 4-(p-amine or p-hydroxy)-phenyl-1-vinyl pyrrolidone, and we have found that this system can be formed by reducing the corresponding succinimide with BH_3-THF.

Studies are being conducted in cooperation with Dr. Edward Harris, (Dartmouth Medical School) on the inhibition of collagenase by our synthetic polymer technique. While the site of attack of the enzyme on collagen is

not known, we have found that poly-L-tyrosine showes a fairly strong inhibition of the enzyme, thus implicating tyrosine in the site of attack. These studies are being continued.

2) Instrumentation:

A mouse shocker and dispenser have been developed and the work is now submitted for publication.

The Bank Chromatograph has been published.

A vacuum valve for coupling the mass spectrometer to a gas chromatograph has been published.

Most of the "bugs" have been eliminated from the timing circuits of the automatic viscometer, and it is clearly evident that good data with a precision of 1 part in 10^5 can be obtained. We have made a preliminary study of the mutarotation of glucose, using this viscometer as the measuring system, and the change in viscosity is clearly evident. The utility of this device in other kinetic studies will be determined.

Significance: Basic to the biochemical, biomedical and organic chemistry programs of the Institute.

Proposed Course of Projects: To continue along the lines indicated above, to include bloodstream leukemias.

Publication: Landis, W. R., Alford, W. C., and Perrine, T. D.: A Slide Valve Suitable for Coupling a Gas Chromatograph to a Mass Spectrometer. Appl. Spectry., 25, 44-46, 1971.

NIAMD-LC-12
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Titles: 1) Chemical Structure - biological activity correlations in the 2'-H-6,7-benzomorphan series.
2) Synthesis of 2'-H-6,7-benzomorphans
3) Synthesis of agonist-antagonists in the benzomorphan series.

Previous Serial Number: Same

Principal Investigator: Arthur E. Jacobson

Other Investigators: W. F. Raub, C. Hansch, H. L. Holmes, G. Thyagarjan

Cooperating Units: NIH (BEMT, RR) (Raub)
Pomona College, California (Hansch)
DRES, Canada (Holmes)

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: 1) To determine whether chemical structure and biological activity (analgetic activity) could be related in the 2'-H-6,7-benzomorphan series of compounds. 2) To use the information derived from (1) in preparing biologically active (agonist-antagonist) 2'-H-benzomorphans.

Methods Employed: The usual organic, instrumental and computer techniques.

Major Findings: The determination of several partition coefficients in the benzomorphan series by C. Hansch and H. L. Holmes resulted in an arithmetic determination of substituent pi values. These pi values (which simulate transport of a compound to a presumed active site) were combined with interatomic steric factors (to simulate inteference with metabolic N-demethylation) to obtain a series of equations. The equations (one

equation/compound) were submitted to computerized regression analysis (formulated by C. Hansch and transferred to DCRT's IBM 360/65 computer by R. Engle, (NCI) and M. Shamos (NCI)). The final equations might prove useful in subsequent synthetic work in the benzomorphan field.

Significance: If regression analysis of equations based on biological data proves successful in the analgetic field, the work should be of interest and significance in allied structure-activity determinations. In the future, it might be feasible and, perhaps, more successful to use a mathematical equation derived from regression analysis for correlation of biological activity with chemical structure rather than the usual intuitive guess, since the former could theoretically lead to separation of the various parameters necessary for biological activity and the latter intuitive guess is not extendable in this manner.

Proposed course of projects: 1) Continuation of structure-activity correlations in the analgetic field. 2) Synthesis of benzomorphans based on data obtained from (1). 3) Synthesis of morphans (in collaboration with H. Ong, E. L. May, and Hoffman-La Roche Pharmaceutical Co.).

Publications:

Mokotoff, M. and Jacobson, A. E.: Azabicyclo Chemistry. II. Synthesis of 1,5-Methano-2,3,4,5-tetrahydro-1H-2-Benzazepines. B-Norbenzomorphans. J. Heterocyclic Chem., 7, 773 (1970).

Kaufmann, S. J., Jacobson, A. E., and Raub, W. F.: Computer-Assisted Data Management in Medicinal Chemistry. I. The Use of a General-Purpose Text Editor. J. Chem. Documentation, 10, 248, 1970.

Jacobson, A. E., Sargent, L. J., and May, E. L.: Chapter 49, "Analgetics" in Medicinal Chemistry, Third Edition, Burger, A. (Ed.), John Wiley & Sons, Inc.-Interscience Publishers, New York 1970.

Eddy, N. B. and Jacobson, A. E.: Agonist-Antagonists. Current Medical Digest, in press, 1971.

Jacobson, A. E.: Structure-Activity Relations of Analgesic and Addictive Potentials. Proceedings of the 15th Annual Meeting of the Eastern Psychiatric Research Association, in press, 1971.

NIAMD-LC-13
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Development of Synthetic Procedures for Alkaloids

Previous Serial Number: Same

Principal Investigator: Edward M. Fry

Other Investigators: None

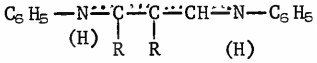
Man Years:
Total: 1
Professional: 1
Others: 0

Project Description:

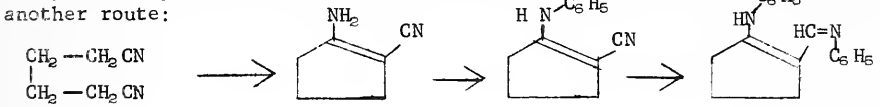
Objectives: The synthesis of nitrogen heterocycles.

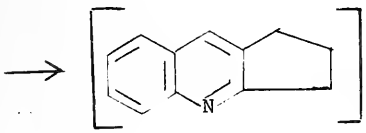
Methods Employed: Standard methods of organic synthesis.

Major Findings: 2,3-Dialkylquinolines derived from iminoamines of the general structure,



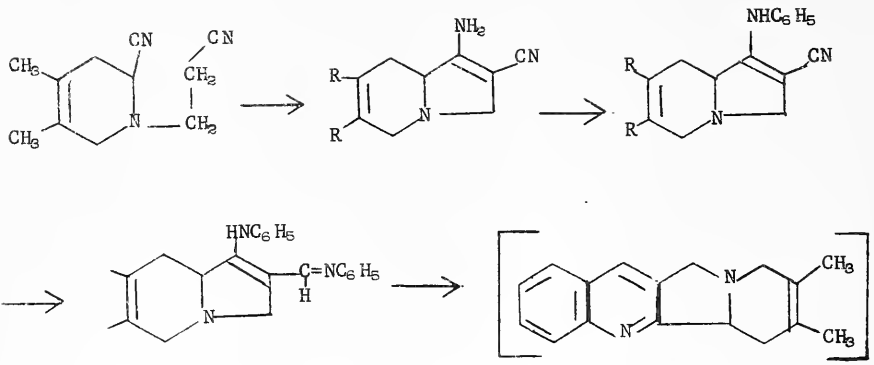
have been obtained in good yields (Gagan and Lloyd, J. Chem. Soc., (C) 1970, 2488). This published work gave impetus to a similar synthesis based on another route:



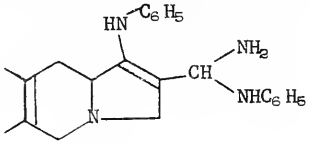


As the iminoamines (penultimate compounds) by both methods are identical, the dinitrili approach is validated.

In like manner:



In contrast to the stability of the cyclopentene analog, the indolizine amine is extremely labile. The presumed intermediate loses ammonia readily,



but the diene is just as readily lost. Conditions favorable for its preparation and ring closure are under investigation.

Publications: Fry, E. M. and Beisler, J. A.: The sodium borohydride reductions of indolylethylpyridinium bromides. Hexahydroindoloquinolizines. J. Org. Chem., 25, 2809, 1970.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Cyclopropyldihydrocodeinone

Previous Serial Number: 7

Principal Investigator: Lewis J. Sargent

Other Investigators: None

Cooperating Units: Pharmacology unit of this Section

Man Years:
Total: 1.0
Professional: 1.0
Others: 0

Project Description:

Objectives: The introduction of a cyclopropane ring at the 7,8-position of codeinone and the determination of the product's analgesic powers relative to those of codeinone and dihydrocodeinone.

Methods Employed: Standard Organic chemical procedures including mass spectrographic techniques.

Major Findings: The reaction of codeinone with an excess of dimethyloxosulfonium methylide in dry tetrahydrofuran led to a product which was separated (via column chromatography) into a small amount of a new codeinone derivative having the expected mass number, along with a quantity of codeine. NMR and IR analyses suggested the presence of an oxirane ring system in the new compound instead of the expected cyclopropyl ketone configuration.

Under normal circumstances, α,β-unsaturated ketones (e.g. codeinone) should react selectively with dimethyloxosulfonium methylide via methyl transfer to the α,β-carbon-carbon double bond to form cyclopropyl ketones. Since it is known that the methylide is also capable of reacting with the carbonyl function of saturated cyclic ketones to form oxiranes, the present anomalous reaction with codeinone to form an oxirane may be due to steric constraints imposed by the morphine ring system. If indeed, the oxirane

ring is present in the new derivative, this should easily be cleaved by lithium aluminum hydride to form a tertiary carbinol at C-6.

Significance: Since a preliminary mouse-assay of the new product has demonstrated better than a 2-fold increase in analgetic activity over codeine, further chemical transformations of this substance should add to the body of knowledge concerned with structure-activity relationships in the morphine series.

Proposed course of project: Preparation of further quantities of the new derivative along with attempts to reductively cleave the oxirane ring to form a tertiary carbinol function at C-6, and eventual assessment of the latter's analgetic activity.

Publication: Sargent, L. J. and May, E. L.: Agonists-antagonists derived from desomorphine and metopon. J. Med. Chem. 13: 1061 1970.

Jacobson, A. E., May, E. L., and Sargent, L. J.: Chapter 49, Analgetics. In Burger, A. (Ed): Third Edition, Medicinal Chemistry. John Wiley & Sons, Inc.-Interscience Publishers, New York 1970.

NIAMD-LC-15
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Asparagine Synthetase and Tumor Inhibitors and Antiinflammatory Agents

Previous Serial Number: Same

Principal Investigator: Everette L. May

Other Investigators: Richard Adamson (NCI) and David Cooney (NCI)

Cooperating Units: National Cancer Institute

Man Years:
Total: 0.25
Professional: 0.25
Other: 0

Project Description:

Objectives: To synthesize asparagine synthetase and tumor inhibitors and study the role of asparagine synthetase in leukemias.

Methods Employed: Usual methods of organic synthesis and analysis; in vitro assays for asparagine synthetase and leukemia inhibition and anti-cancer assays in mice.

Major Findings: 1,4-Bis-(1-piperidino)-2-amino-butane (I) and its N-methyl (II) and N,N-dimethyl derivatives were synthesized from N-carbobenzoxy-L-aspartic acid for comparison with corresponding phenethylamino (III) congeners which had been shown (see 1970 report) to have antileukemic properties. Compound III was equal to indomethacin in inhibiting the exudate formation in the croton-oil, granuloma pouch technique; I and II were more effective than oxyphenbutazone.

Significance: This research is consonant with program aims to discover improved chemotherapeutic agents. The antiinflammatory activity of the strongly basic substances appears to provide a new lead. Hitherto only acidic compounds which tend to be ulcerogenic have been effective.

Proposed Course of the Project: To further evaluate compounds already prepared and to synthesize new congeners including small peptides of asparagine and aspartic acid with guidance from test results.

Publications:

Ong, H. and May, E. L.: Photocyclizations. I. 4,5-Dihydro-1H-Naphth[1,8-de]azocin-2[3H]-One. J. Org. Chem., 35, 2544, 1970.

Jacobson, A. E., Sargent, L. J., and May, E. L.: Chapter 49, "Analgetics" in Medicinal Chemistry, Third Edition, Burger, A. (Ed.), John Wiley & Sons, Inc.-Interscience Publishers, New York 1970.

Sargent, L. J. and May, E. L.: Agonists-Antagonists Derived from Desomorphine and Metopon. J. Med. Chem., 13, 1061, 1970.

Takeda, M. and May, E. L.: Acyl Derivatives of 5-Hydroxy-6,7-benzomorphans. Prodine Congeners. J. Med. Chem., 13 1223, 1970.

May, E. L. and Takeda, Mikio: Optical Isomers of Miscellaneous Strong Analgetics. J. Med. Chem., 13, 805, 1970.

NIAMD-LC-16
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Evaluation of Compounds for Analgesic Activity and Dependence Liability

Previous Serial Number: Same

Principal Investigators: Everette L. May and Lewis J. Sargent

Other Investigators: Nathan B. Eddy, Consultant

Cooperating Units: Animal Production Section, NIH; Department of Pharmacology, University of Michigan

Man Years:
Total: 1.5
Professional: 0.5
Other: 1

Project Description:

Objectives: To test substances for analgesic activity, abuse liability and narcotic antagonist activity and to serve in an advisory capacity to the Bureau of Narcotics and Dangerous Drugs, FDA, WHO, and the pharmaceutical industry.

Methods Employed: Eddy hot-plate and Nilsen (electrical stimulus to the tail) methods of analgesic assay and 24-hr-acute toxicity determinations CDGP mice). Abuse liability and antagonistic properties were assessed in monkeys and, if warranted, in man at the Addiction Research Center, Lexington, Kentucky.

Major Findings: A total of 87 new drugs were assessed for analgesic activity. Sixty-seven of these were subjected to complete assay by the hot-plate method, 27 by the newly installed Nilsen technique. Some 40 of these were deemed of sufficient interest for study in monkeys for dependence liability and two were submitted for trials in man for abuse liability and

pain-relief efficacy. Several acute 24-hour toxicity experiments were performed on pure compounds and on some steroid extracts. The Nilsen method has proved to be a good complement to the hot-plate technique in testing as a safeguard against false negatives.

Significance: Aids the research of the chemistry unit, LC; the pharmaceutical industry and university laboratories engaged in research on CNS type compounds. Also enhances the section's function in an advisory capacity on drugs of abuse.

Proposed Course of Project: To continue essentially unchanged.

Publications: Eddy, N.B. and Martin, W. R.: Drug Dependence of Specific Opiate Antagonist Type. Pharmakopsychiat. Neuro-Psychopharmak., Vol. 3 No. 2, 73-82, 1970.

Eddy, N. B. and Jacobson, A. E.: Agonists-Antagonists Current Medical Digest (in press) 1971.

NIAMD-LC-17
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Synthetic Approaches to 9-Methyl-6,7-benzomorphans and their Derivatives

Previous Serial Number: Same

Principal Investigator: G. Thyagarajan (V.S.) and E. L. May

Other Investigators: None

Cooperating Units: Pharmacology Unit and Section on Microanalytical Services and Instrumentation of this Laboratory

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: To synthesize 9-methyl-6,7-benzomorphans and derivatives by known or new routes and evaluate their analgesic activity.

Methods Employed: Reaction methods and techniques generally employed in organic chemistry including modern chromatographic and spectrometric tools.

Major Findings: 1) Synthesis from 3-picoline. The improved, structurally definitive synthesis of 2-benzyl-1,2,5,6-tetrahydropyridines (precursors of analgetic 6,7-benzomorphans) disclosed in the last report (1969-1970) was applied for synthesis of some more members to demonstrate its general applicability. Thus, 2-benzylpyridine (I), 2-*p*-methoxybenzylpyridine (II), 2-*p*-methoxybenzyl-4-methylpyridine (III), and 2-*p*-methoxybenzyl-5-methylpyridine (IV) were converted to the respective 1,2,5,6-tetrahydropyridines in improved yields. In the applicable cases these were cyclized to the corresponding known 6,7-benzomorphans. The mild conditions employed in the $NaBH_4$ reduction step led to smooth distillation and improved yields of the

tetrahydro derivatives. The new sequence was successfully employed to synthesize 1-benzyl-3,4-dimethyl-2-p-methoxybenzyl-1,2,5,6-tetrahydropyridine (V), a preferred intermediate for an improved preparation of the potent analgesic Pentazocine. 2) Synthesis from 4-phenyl-3-methylpyridine. The proposed synthesis involves an 8-step sequence to convert 4-phenyl-3-methyl-pyridine (VI) to 2,9-dimethyl-6,7-benzomorphan.

Continuing the synthetic sequence outlined earlier, 1,3-dimethyl-4-phenyl-4-piperidinol (VII) was dehydrated to 2 3:1 mixture of Δ^3- and Δ^4-tetrahydro-1,3-dimethyl-4-phenyl pyridines. Aromatization of this mixture to 4-phenyl-3-picoline (VI) was achieved in 70% yield employing 5% Pd-on-alumina catalyst. VI afforded a nearly quantitative yield of its N-oxide (VII) which was converted via its N-methoxy-methosulphate to 2-cyano-3-methyl-4-phenylpyridine (VIII) in 90% yield. However, conversion of VIII to 2-carbomethoxy-3-methyl-4-phenylpyridine (IX) has not been successful so far owing to resistance of the 2-cyano group to hydrolysis. Refluxing in methanolic HCl, alkaline hydrolysis in aqueous solution using Amberlite IRA 400 or use of acetone-H_2O_2 did not give the desired product. An effective way to accomplish this hydrolysis is being explored.

Significance: The improved synthesis of 2-benzyl 1,2,5,6-tetrahydro-pyridines from commercially accessible starting materials developed in the course of this work offers broad potential for the unambiguous synthesis of specifically substituted 6,7-benzomorphans.

Proposed course of the project: To continue until α- and β-2,9-dimethyl-2'-hydroxy-6,7-benzomorphans and derivatives have been synthesized and pharmacologically evaluated.

Publications: Thyagarajan, G. and May, E. L.: Improved Synthesis of 2-benzyl-1,2,5,6-tetrahydropyridines, Precursors of Analgetic 6,7-Benzomorphans. J. Heterocyclic Chem., in press, 1971.

NIAMD-LC-18
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Aldolase Inhibitors

Previous Serial Number: 8

Principal Investigators: James G. Murphy

Other Investigators: None

Cooperating Units: None

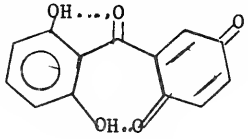
Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

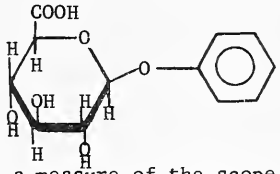
Objectives: Specific objectives include the finding of new substances for biological, pharmacological and possible clinical evaluation in inflammatory and tumorigenic conditions.

Methods Employed and Major Findings: A. Using the method of oxidative phosphonylation, dihydroxyacetone-O-phosphonate, glyceraldehyde-O-phosphonate and fructose di-O-phosphonate were isolated in approximate yields of 47%, 33%, and 61%, respectively. They were isolated as their magnesium salts which are water soluble and neutral solids.

B. From the reaction of 2,5-dihydroxy-benzoic acid and resorcinal there was isolated a high melting, fine yellow needle product. Based on the method of synthesis, its mass spectrum and diagnostic infrared absorption peaks it is designated 2-(2′,2′-dihydroxy-benzoyl)-1,4-benzoquinone, a structure rigidified by hydrogen bonding and which because of analogies to the salicylates may have value as an antiinflammatory agent.



C. Glucuronidation, a natural pathway of detoxification, gives rise to a variety of glucuronides. Besides this basic biochemical aspect, a further interest in glucuronides arises from recent work which has shown that they are capable of selectively blocking metabolic processes. The glucuronides, however, apart from isolation from natural sources, are generally available only from the Helferich or the Koenigs-Knorr syntheses, both of which are tedious and limited. Two alternate routes to the glucuronides have been found as exemplified in the synthesis of phenyl-B-D-glucopyranouronic acid.



Further examples to obtain a measure of the scope of the reaction are planned.

Significance: The above research makes new substances available for pharmacological evaluation.

Proposed Course of Project: Evaluation of the pharmacology of 2-(2′,-2′-dihydroxybenzoyl)-1,4-benzoquinone and closely related materials for antiinflammatory action and extending the scope of the glucuronidation reaction.

Publications: None

NIAMD-LC-19
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Activities at the United Nations Narcotics Laboratory, October to March

Previous Serial Number: None

Principal Investigator: Everette L. May

Other Investigators: Olav Braenden, E. Lumsden, I. Chilov, M. L. Ibanez

Cooperating Units: University of Athens Medical School

Man Years:
Total: 0.5
Professional: 0.5
Other: 0

Project Description:

Objectives: To examine Cannabis samples for alkaloids and to perform experiments in general leading toward a more comprehensive analysis of Cannabis, particularly products resulting from combustion and smoking. To correlate the results with pharmacology.

Methods Employed: Thin-layer, column and gas chromatographic techniques combined with usual spectral examinations.

Major Findings: Only traces of tertiary-nitrogen alkaloids were detected in each of three different Cannabis samples (one a reference sample composed of leaves seeds and stems of plants widely distributed) by several different extraction techniques. The alkaloids were separated into 2-5 distinct components by thin-layer chromatography. Much more material would have to be extracted for accumulation of sufficient amounts for structure study and/or pharmacological assay. It is possible, of course, that other species and origins of Cannabis are more abundant in alkaloidal content.

The best and most exhaustive extraction solvents were found to be methanol followed by petroleum ether. The resultant extract could be separated into 15-30 distinct cannabinoids (depending on the origin of the sample) by a combination of column and thin-layer chromatography. The lesser components could be sufficiently concentrated on a silica-gel column for further purification on thick-layer plates and subsequent structure determination. Temperature-programmed gas chromatography showed up to 50 components, most of them present in very small amounts.

During the course of examination of various cannabis extracts, it was discovered that:

1. Dilute, cold Na_2CO_3 solution is superior to KOH or $NaHCO_3$ for extraction of cannabinoid acids.

2. The quantitative estimation of cannabinoids by the glpc method of Lerner and Lerner appears valid for combined Δ^9 and Δ^8-tetrahydrocannabinol and cannabinol but not for cannabidiol, because cannabidiolic acid undergoes decarboxylation under the conditions of the experiment to give cannabidiol.

3. Each cannabis extract gives its own characteristic ir spectrum in chloroform, an aid in the determination of origin.

4. $CHCl_3$-Et_2NH (95:5), C_6H_6-$CHCl_3$-Et_2NH (50:45:5 or 80:15:5 depending upon the chromatoplates used) are superior solvent systems for 8-12 of the less polar components of Cannabis and that 85:15 or 9:1 C_6H_6-EtOH is superior for the acids and other more polar constituents.

5. A sample brought to the United Nations and claimed by Professor Miras of the University of Athens to be very psychoactive on ingestion by smoking was shown to contain only traces of tetrahydrocannabinol but substantial amounts of several other cannabinoids including cannabidiol and cannabidiolic acid.

Significance: The efforts being expended at the U.N. Narcotics Laboratory in Cannabis activities, and thus the small contribution recorded herein should aid in the world-wide research program to determine the complete pharmacologic profile of various cannabis resins and various constituents including the presumed principally psychoactive ingredients Δ^9- and Δ^8-tetrahydrocannabinols. This will in turn aid in decisions pertaining to possible medical benefits and measures of control to be applied.

Proposed Course of Project: It is intended to continue to help the United Nations and the National Institute of Mental Health to coordinate and foster the various, world-wide research programs now in progress.

Honors and Awards: Served as a consultant to the U. N. Secretariat at the Plenipotentiary Conference for Consideration of a Protocol for the International Control of Psychotropic Substances held in Vienna January 11 to February 21.

Serial No. NIAMD-LC-20
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Selective Modification of Free and Bound Tryptophan

Previous Serial Number: SAME

Principal Investigator: Thomas F. Spande

Other Investigators: H. C. J. Ottenheym, B. Witkop and George Glenner, NIAMD-LEP

Cooperating Units: None

Man Years:

Total: 1.8
Professional: 1.8
Others: 0

Project Description:

Objectives: 1. To determine whether tryptophan derivatives and simple indoles will react with diazonium salts commonly used in histochemical diagnostic tests.

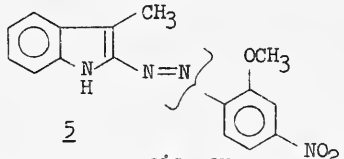
2. To determine the structure(s) of any resulting reaction products.

3. To investigate the reaction of tryptophan in proteins with diazonium salts.

Methods Employed: NMR, UV and IR spectroscopy, mass spectrometry and thin layer and column chromatography.

Major Findings: 1. Diazonium salts such as p-nitrobenzene diazonium chloride (1) and o-methoxy-p-nitrobenzene diazonium chloride (2) do react with skatole (3) (3-methylindole), N-acetyl tryptophan ethyl ester (4) and ethyl indole-3-propionate at 0° or R.T. When the reactions are conducted in 50% aqueous dioxane at pH 7.0, the major byproducts are nitrobenzene from 1 and m-nitroanisole from 2, resulting from reduction of the diazonium salts by the dioxane. The reaction with the indoles is fairly slow under these conditions but may be driven to completion in several days using 3-4 fold excesses of reagent. The use of dioxane as a solvent largely prevents radical reactions which might result from decomposition of the diazonium salts in a non-reducing medium. Even so, traces of a biphenyl derivative were isolated in the reaction of 4 with 2.

2. Skatole gives a single product with 2 which can be purified easily by crystallization. The mass spectrum and IR spectrum support structure 5 below.



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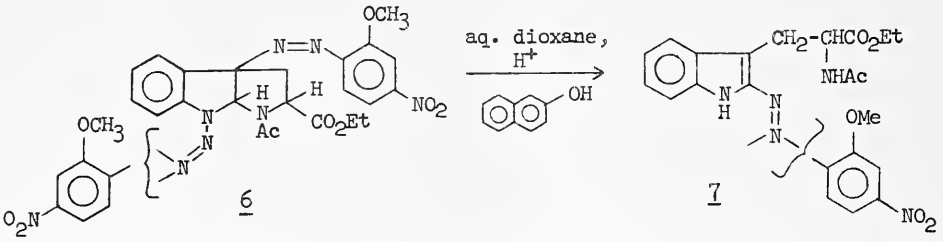
cis- or trans- ?

3. The reaction of 2 with 4 is considerably more complex. Four products account for 81% of the starting material. The two major products are closely related bis-substituted derivatives, while the two minor components are mono-substituted derivatives. Although more work remains to be done to confirm the structures, we feel that the bis-substituted derivatives have structures represented by 6 and differ in being cis and trans isomers with respect to the

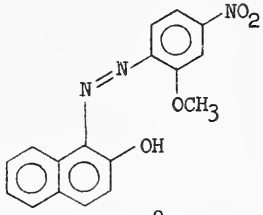


6

cis- and trans-

Total yield 57%

7



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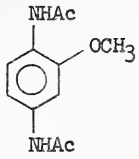
triazene azo-linkage. Either of the bis-substituted isomers can be converted with dilute acid in aqueous dioxane containing β-naphthol or N,N-diethylaniline to the principal 1:1 reaction product 7 (produced initially in 24% yield). The bis-labeled materials have very similar chromatographic behavior, NMR, IR and UV spectra, both turn a deep cherry red on TLC when sprayed with 1 N HCl and one isomer can be converted to the other by exposure to dilute acid. Neither isomer gives a parent ion in the mass spectrometer; however, both show as the highest significant peak an ion at m/e = 452. Both 1:1 complexes, on the other hand, give true parent ions at m/e = 453. The 1:1 complexes presumably are 2-substituted indoles--as shown in structure 7--and may well be again

cis- and trans-isomers. The UV spectrum of the minor 1:1 component (ca. 5%) resembles very closely that of the skatole product (460 mμ) whereas the major 1:1 product (24% yield) is quite different (410 mμ). The latter material has been reduced at pH 8 with $Na_2S_2O_4$ and acetylated to give two materials--one has been shown to be 9, the other a yet unidentified acetylated amino indole.



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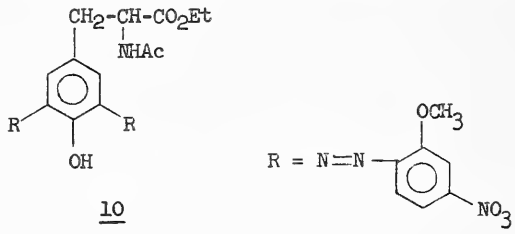
4. While it is well-known that diazonium salts react with proteins to form azo-linkages with tyrosine and histidine residues, the possibility of reaction with tryptophan has only been speculated upon. We find that a gramicidin containing only tryptophan among its aromatic residues, does react at pH 4 or 7 with 2 to give orange-red proteins absorbing at 410-420 mμ and lacking the usual indole chromophore in the ultraviolet region. Lysozyme, at pH 4, gives a similar spectrum, while at pH 7, the visible spectrum is more complex. Addition of alkali gives rise to an absorption peak at around 550 mμ. The same absorption is noted with the N-acetyl-bis-azo tyrosine ethyl ester model compound 10. A tentative conclusion is that at pH 4, only reaction with



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tryptophan (or possibly histidine) occurs while at pH 7, reaction with tyrosine occurs as well. At pH 7, in the presence of 6 M guanidine hydrochloride, the reaction with tyrosine is suppressed, due to the faster reaction of reagent with guanidine than with tyrosine.

Significance to Biomedical Research and the Program of the Institute: The finding that tryptophan in proteins can react readily with diazonium salts has significant implications in histochemical diagnostic tests. We have conducted our studies with the so-called "Fast Red-B," one of the most widely used diazonium salts. The use of diazonium salts as affinity-labeling reagents is currently providing much useful information on the combining sites of antibodies. It is generally assumed that reaction can occur only with tyrosine or histidine; however, our preliminary studies indicate the great likelihood

of reaction also with tryptophan residues at the combining site.

Proposed Course: To complete the structure proof of the 1:1 complexes with 4. In particular, 3 and 4 labeled with deuterium in the 2-position will be coupled with 2. To explore the possibility that diazo coupling and dithionite reduction can be used as a practical synthesis of aminoindoles--an uncommon indole substituent.

Honors and Awards: None

Publication:

Kuttan, R., Radhakrishnan, A. N., Spande, T., and Witkop, B.: sym-Homospermidine, a naturally occurring polyamine. Biochem. 10: 361-365, 1971

Serial No. NIAMD-LC-21
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biosynthesis of Collagen: Inhibitors of Proline Hydroxylase; New Synthetic and Natural Analogs of Hydroxyproline

Previous Serial Number: SAME

Principal Investigator: B. Witkop

Other Investigators: NONE

Cooperating Units: S. Udenfriend, Roche Institute of Molecular Biology, Nutley, New Jersey
A. Verbiscar, Institute of Drug Design, Inc., Sierra Madre, California
Isabella Karle, U. S. Naval Research Laboratory, Washington, D. C.

Man Years

Total: .5
Professional: .3
Other: .3

Project Description:

Objectives: A rapid assay method for proline hydroxylase developed in the laboratory of Dr. S. Udenfriend has made possible a program on selective inhibitors of proline hydroxylase. Proline analogs have been incorporated into oligopeptides.

Methods Employed: Thin layer chromatography, silica gel and ion exchange column chromatography, paper chromatography, and isotope techniques were used for the purification of various intermediates and for the identification of products. Displacement of tosyloxy, mesyloxy, and nisyloxy groups of suitable hydroxyproline derivatives by hydride, deuteride and tritide ion gave selectively labeled and sterically homogeneous L-proline derivatives.

Major Findings: A good substrate for proline hydroxylase has now been found in the peptide hormone bradykinin: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg.
↑
The point of attachment of the hydroxyl group is the third amino acid in the sequence, namely, the second proline. When this proline is replaced by 3,4-dehydro-L-proline one obtains a nonapeptide which now acts as an inhibitor of

proline hydroxylase. In spite of this inhibition there is still consumption of cofactor, namely, conversion of β-ketoglutarate to succinate.

Significance to Bio-medical Research and the Program of the Institute: The mechanism of formation of hydroxyproline, an amino acid found only in the protein collagen, in large amounts, is of biochemical, therapeutic and even clinical interest. Urinary hydroxyproline levels permit to diagnose a number of syndromes and metabolic disorders, such as Marfan's syndrome, chondrosarcomas, acromegaly, etc.

Proposed Course: Further analogs of bradykinin are now being synthesized. The effect of such inhibitors in scar tissue formation, wound healing and cicatrization will be investigated.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-22
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Synthesis of Potentially Antiviral and Carcinostatic Nucleotides and Nucleosides. Chemistry of Irradiation Damage to DNA.

Previous Serial Number: SAME

Principal Investigator: P. Torrence

Other Investigators: B. Witkop, T. Nagamachi, Sam Baron, NIAID-LBV, Maxine Singer, NIAMD-LBM

Cooperating Units: Professor Lawrence Grossman, Brandeis University; Florence White, NCI-DE; Gerald A. LePage, Stanford Research Institute, Palo Alto

Man Years

Total: 2.0
Professional: 1.8
Other: 0.3

Project Description:

Objectives: Procedures are being developed to synthesize what may be "fraudulent" nucleosides and nucleotides. Of special interest, aside from the unusual chemistry of such compounds, are the biological properties of such relatively subtle structural variations, particularly in reference to their behavior as enzyme substrates, the triplet of the genetic code, the physical properties of polynucleotides and interferon induction.

Methods Employed: Thin-layer, paper, ion-exchange and column chromatography, liquid scintillation, ultraviolet, visible, infrared, nuclear magnetic resonance, optical rotatory dispersion and mass spectroscopy.

Major Findings: 1. [α-P^{32}]-Cyclopropyluridine-5'-diphosphate and [5-^{14}C]-dihydrouridine-5'-diphosphate have been prepared using chemical methods.

2. Both dihydrouridine-5'-diphosphate (H_2UDP) and cyclopropyluridine-5'-diphosphate (ΔUDP) are equally poor inhibitors of the enzyme polynucleotide phosphorylase.

3. Neither H_2UDP nor ΔUDP is a substrate for polynucleotide phosphorylase (PNPase) when either compound is tested by itself using magnesium or manganese as metal cofactor.

4. When $(A_p)_3A$ is used as a primer for the polymerization reaction, both H_2UDP and ΔUDP undergo polymerization, the maximum occurring at a one-to-one ratio of substrate to primer. Indications are that the products result from the addition of H_2UDP or ΔUDP to the primer.

5. Both H_2UDP and ΔUDP can undergo copolymerization with UDP, CDP, IDP or ADP. The amount of H_2UDP or ΔUDP incorporated varies with the initial substrates ratio as well as with the metal ion, but in all cases H_2UDP is at least twice as effective as ΔUDP as a substrate in the copolymerization.

6. The polymeric products from the copolymerization reaction have been deproteinized and purified by chromatography on Sephadex G-75. A copolymer consisting of U and [^{14}C]-H_2U is degraded by Pancreatic Ribonuclease to give products which indicate that the H_2U residues are internal in the polynucleotide chain as well as terminal. Preliminary indications are that the copolymer formed from ΔUDP and CDP or UDP is of the same nature.

7. Purified samples of poly(C, H_2U) with C/H_2U = 100/5 and poly(C, H_2U) with C/H_2U = 2/1 have been submitted to Dr. Sam Baron for testing as to their ability to induce interferon, either by themselves or annealed with poly I.

Significance to Biomedical Research and the Program of the Institute: These "fraudulent" nucleosides are of substantial interest as potential antivirals, immuno-suppressive or carcinostatic agents and as tools for examining the active site of enzymes important in nucleic acid metabolism. Their potential usefulness is further expanded by polymerization to polynucleotides and thence used to induce interferon or for their immune stimulating effect. With regard to the former, such analogues may be useful in elucidating factors governing interferon production as well as leading to less toxic, more ribonuclease-resistant forms of poly I·poly C.

Proposed Course: Present work is being directed to obtaining additional information on the conformation of ΔUDP since this may shed light on the nature of the active site of PNPase. The physical properties of copolymers of H_2UDP or ΔUDP with normal nucleoside diphosphates will be examined to determine the effect of the insertion of the cyclopropane ring in polynucleotides. Further characterization of the copolymer poly(C, ΔU) will be carried out and its interferon-inducing ability determined. The photoproduct of ΔU, which is the first seven-membered ring analogue of UDP, will be converted to its diphosphate and studied as a substrate for PNPase.

Honors and Awards: None

Publications:

Kunieda, T. and Witkop, B.: Selective N-methylations of heterocycles with dimethyloxosulfonium methylide. J. Org. Chem. 35: 3981-3982, 1970.

Kunieda, T. and Witkop, B.: Preparation and photochemistry of pyrimidine nucleoside sulfonium ylides. J. Am. Chem. Soc., in press.

Kunieda, T. and Witkop, B.: Photo- and stereochemistry of 5,6-methylene-pyrimidine nucleosides, bicyclic isomers of thymidine, and 5-methyluridine. J. Am. Chem. Soc., in press, 1971.

Kunieda, T. and Witkop, B.: Hydrogenolysis and stereochemistry of photo-dimers of thymine and thymidine. J. Am. Chem. Soc., in press.

Serial No. NIAMD-LC-23a
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Inhibition of Sodium-Potassium Dependent Adenosine Triphosphatase by Fluorescent Derivatives of Strophanthidin and by Steroidal Alkaloids

Previous Serial Number: NIAMD-LC-23

Principal Investigators: F. Oesch and J. Daly

Other Investigators: J. A. Waters and B. Witkop

Cooperating Units: R. W. Albers, NINDS-LNC

Man Years

Total: .5
Professional: .3
Other: .3

Project Description:

Objectives: To study the inhibitory activity of Na^+/K^+-dependent ATPase by (A) fluorescent strophanthidin derivatives and (B) other membrane depolarizing agents.

Methods Employed: Strophanthidin derivatives, veratridine analogs and batrachotoxin A p-bromobenzoate and their parent compounds were tested as inhibitors of Na^+/K^+-dependent ATPase, measuring the rate of formation of ADP by recording the rate of NADH disappearance spectrophotometrically in the presence of excess phosphoenolpyruvate, pyruvokinase and lactic dehydrogenase.

Major Findings: (A) The strophanthidin 3-O-esters showed very strong Na^+/K^+-dependent ATPase inhibitory activities (appreciably stronger than ouabain) whereas the strophanthidin-19-hydrazones were less active. One of the most active inhibitors of this latter group, the strophanthidin 19-salicylhydrazone, showed strong fluorescence when irradiated with UV light. (B) Other membrane depolarizing agents such as veratridine, batrachotoxin and related compounds showed no or very weak Na^+/K^+-dependent ATPase inhibition.

Significance to Biomedical Research and the Program of the Institute: Fluorescent inhibitors of Na^+/K^+-dependent ATPase inhibitors should provide powerful tools as fluorescent probes of the enzyme in studying the active

site of Na^+/K^+-dependent ATPase and the molecular action of such drugs in their effect on ion transport and on membrane receptors.

Proposed Course: To study the effect of binding to the enzyme on the fluorescent properties of the molecules.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-23b
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Pyrrole Esters of Various Alkaloids and Steroids

Previous Serial Number: NIAMD-LC-23

Principal Investigator: J. A. Waters

Other Investigators: B. Witkop and J. Daly

Cooperating Units: None

Man Years

Total: .5
Professional: .3
Other: .3

Project Description:

Objectives: To synthesize pyrrole esters (similar to the pyrrole ester found in batrachotoxin) of various alkaloids and steroids for pharmacological evaluation.

Methods Employed: 2,4,5-Trimethylpyrrole-3-carboxylic acid esters of various alkaloids and steroids possessing a secondary hydroxyl group have been prepared using trifluoroacetic anhydrides as the acylating agent.

Major Findings: Esterification of veracevine, jervine and methyl reserpate with the labile pyrrole carboxylic acid has been achieved, which apparently involves a mixed trifluoroacetic-pyrrole carboxylic anhydride intermediate. Esterification of other alkaloids and steroids by this method, i.e., scopoline, codeine, morphine, cholesterol and strophanthidin is now in progress. Conventional methods of esterification resulted in the decomposition of the pyrrole moiety.

Significance to Biomedical Research and the Program of the Institute: These compounds may exhibit either enhanced or dissimilar pharmacological activities from the parent alkaloids and steroids, and they should be useful in studying the activity at the molecular level.

Proposed Course: To study in detail the mechanism involved in the formation of these pyrrole esters, to extend the reaction to aromatic, alkyl, and cycloalkyl carboxylic acids, and to evaluate the drugs for pharmacological activity.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-23c
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Reductive Cleavage of the Oxide Bridge of Veratridine and Related Steroidal Alkaloids

Previous Serial Number: NIAMD-LC-23

Principal Investigator: J. A. Waters

Other Investigators: J. Daly and B. Witkop

Cooperating Units: None

Man Years:

Total: .5
Professional: .3
Others: .3

Project Description:

Objectives: To open the oxide bridge of veratridine and related compounds without altering the other functional groups within the molecule (e.g., the 3,4-dimethoxybenzoyl moiety of veratridine) and to evaluate the physiological activity of these compounds.

Methods Employed: Various metal hydride reagents have been studied which conceivably could reductively cleave the 4,9-oxide bridge of veratridine. Sodium borohydride in methanol (2-propanol) and sodium borohydride-diborane gave negative results, while lithium aluminum hydride in refluxing dioxane gave a desired veratridine analog.

Major Findings: Infrared and mass spectral data of the lithium aluminum hydride-veratridine compound suggest a 4,9-diol-5-ene was produced without removing the 3-veratroyl ester group. The mechanism involved can be explained by hydride ion attack at C-4 with concomitant β-elimination of a C-6 hydrogen atom.

Significance to Biomedical Research and the Program of the Institute: Whereas prior studies concerning the base-promoted opening of the oxide bridge of cerevatrum alkaloids are accompanied by hydrolysis of ester groups, this method affords compounds with intact ester groups which are necessary for the maintenance of pharmacological activity. This study will determine what

effect the oxide bridge has in regard to pharmacological activity and site of action of these alkaloids.

Proposed Course: To fully elucidate the structure of this veratridine analog and to study its biological activity in direct comparison with the parent compounds.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-24
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Photochemistry of Free and Bound Nucleotides

Previous Serial Number: Same

Principal Investigator: Yoshikazu Kondo

Other Investigators: Bernhard Witkop and Jean-Louis Fourrey

Cooperating Units: Prof. S. Y. Wang, Johns Hopkins University

Man Years

Total: 1.0
Professional: .8
Other: .3

This project has been terminated.

Publications:

Kondo, Y., Fourrey, J.-L., and Witkop, B.: Alkali-sensitivity of 3-methyl-pyrimidine nucleosides. J. Am. Chem. Soc., in press.

Serial No. NIAMD-LC-25
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Photochemistry of Pharmacodynamic Amines

Previous Serial Number: SAME

Principal Investigator: Bernhard Witkop

Other Investigators: NONE

Cooperating Units: O. Yonemitsu, Y. Kanaoka and Y. Okuno, University of Hokkaido, Sapporo, Japan
I. L. Karle, U. S. Naval Research Lab., Washington, D.C.
George Hammond, Thomas McGuire, California Institute of Technology, Pasadena, California
A. Verbiscar, Institute of Drug Design, Inc., Sierra Madre, California

Man Years:

Total: .5
Professional: .3
Other: .3

Project Description:

Objectives: Reactions for the selective modification of simple amino acids in proteins are needed for the study of the composition and function of their active sites. Although extensive interest to develop such reactions has been shown by many investigators. photochemical methods were applied only recently to this problem. Photochemical methods have been elaborated which make it possible to deliver selectively a distinct amount of energy to a system. The photochemical behavior of free and bound aromatic amino acids and the corresponding pharmacodynamic amines and certain of their derivatives therefore have been investigated.

Methods Employed: Ultraviolet irradiation, thin-layer chromatography, silica gel and ion exchange column chromatography, paper chromatography and electrophoresis, UV, IR, NMR and mass spectroscopy.

Major Findings: The fluorescence of many electron-rich aromatics is quenched efficiently by methyl chloroacetate and chloroacetamide. The absorption spectra of the quenchers show no transition above 280 nm; thus no low-

lying singlet states are available for classical excitation transfer. The absorption spectra of the aromatics in acetonitrile were unchanged by the addition of either methyl chloroacetate or chloroacetamide. The unquenched portion of the fluorescence has the same spectral distribution as that in solutions without quencher. It is of considerable interest that methoxy substitution, which presumably increases electron density of the aromatic nucleus, leads to more efficient quenching. Quenching rate constants are dependent upon solvent polarity. Absorption and emission properties of N-acetyltryptamine (I) and N-chloroacetyltryptamine (II) are essentially identical. However, the intensity of room temperature fluorescence of I is approximately 15 times that of II. Fluorescence from N-chloroacetyl-3,4-dimethoxyphenethylamine has also been found to be significantly less than that from N-acetyl-3,4-dimethoxyphenethylamine. This behavior is most certainly due to intramolecular singlet quenching by the chloro-substituted amide. When an aqueous ethanol solution of N-chloroacetyl-3,4-dimethoxypropylamine (1) was irradiated with a high-pressure mercury lamp (100 W) in an atmosphere of nitrogen for 3 hr, the photocyclization product, 8,9-dimethoxy-1,2,3,4,5,6-hexahydro-3-benzazocin-2-one (2), mp 215°, was isolated in 8% yield. The structure of 2 was confirmed in analogy to that of 7,8-dimethoxy-1,2,4,5-tetrahydro-3H-3-benzazepin-2-one by mass and nmr spectroscopy. Another crystalline product, mp 122-124°, isolated by silica-gel chromatography, has a new carbonyl group (1730 cm^{-1}) in addition to the original amide (1625 cm^{-1}) and no NH group in the ir spectrum. This compound was shown to be 1-methoxy-4-azatricyclo-[6,2,2,$0^{4,8}$]dodeca-11-ene-3,10-dione. Photolysis of N-chloroacetylphenylbutylamine under the same condition gave the nine-membered lactam, 9,10-dimethoxy-1,2,4,5,6,7-hexahydro-3H-3-benzazonin-2-one, mp 178-179°, in 13% yield.

Significance to Biomedical Research and the Program of the Institute: The application of photochemical methods to aromatic amino acids and related pharmacodynamic amines and their derivatives affords new heterocyclic systems, new routes to compounds of biological interest and candidates for evaluation of CNS-activity.

Proposed Course: Fluorescence quenching studies are now in progress in collaboration with the country's leading mechanical photochemist, Professor George Hammond, California Institute of Technology, to determine the mechanism of these fascinating photocyclizations.

Honors and Awards: Paul Karrer Medal, Paul Karrer Lecture, University of Zurich, 1971

Publications:

Yonemitsu, O., Okuno, Y., Kanaoka, Y., and Witkop, B.: Photocyclization of pharmacodynamic amines. IV. Novel heterocycles from N-chloroacetyl-3,4-dimethoxyphenethylamine. J. Am. Chem. Soc. 92: 5686-5690, 1970.

Yonemitsu, O., Nakai, H., Kanaoka, Y., Karle, I. L., and Witkop, B.: Photocyclizations of pharmacodynamic amines. V. Unusual rearrangements of the mescaline skeleton. J. Am. Chem. Soc. 92: 5691-5700, 1970.

McCall, M. T., Hammond, G. S., Yonemitsu, O., and Witkop, B.: Mechanisms of photochemical reactions in solution. LXV. Quenching of excited singlet states of electron-rich aromatic compounds by methyl chloroacetate. J. Am. Chem. Soc. 92: 6991-6993, 1970.

Serial No. NIAMD-LC-26
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: New Natural Amino Acids: Synthesis of L-Bicyclo[2.1.0]-1-azahexane-2-carboxylic Acid from Horse Chestnuts

Previous Serial Number: SAME

Principal Investigator: Y. Fujimoto

Other Investigators: B. Witkop and F. Irreverre, NIAMD-LBC-1

Cooperating Units: S. Udenfriend, Roche Institute of Molecular Biology, Nutley, New Jersey

Man Years

Total: .8
Professional: .5
Other: .3

This project has been terminated.

Publications:

Fujimoto, Y., Irreverre, F., Karle, J. M., Karle, I. L., and Witkop, B.: Synthesis and X-ray analysis of cis-3,4-methylene-L-proline, the new natural amino acid from horse chestnuts, and of its trans-isomer. J. Am. Chem. Soc., in press.

Serial No. NIAMD-LC-27
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Batrachotoxin, Pumiliotoxin, Histrionicotoxin A and Other Physiologically Active Compounds from Amphibian Skins

Previous Serial Number: NIAMD-LC-18

Principal Investigator: Bernhard Witkop

Other Investigators: J. A. Waters, J. Daly and D. Johnson

Cooperating Units: Charles W. Myers, American Museum of Natural History, New York, N. Y.;
E. X. Albuquerque, State University of New York at Buffalo;
Isabella Karle, U. S. Naval Research Laboratory, Washington, D. C.;
G. Habermehl, Technische Hochschule, Darmstadt, Germany;
T. Tokuyama, Osaka City University, Osaka, Japan

Man Years:

Total: .3
Professional: .3
Others: 0

Project Description:

Objectives: To elucidate the molecular basis for the pharmacological activity of batrachotoxin, tetrodotoxin, histrionicotoxin and related substances. To isolate and elucidate the structures of other pharmacologically active substances found in skin extracts of neotropical frogs of the family Dendrobatidae. To explore biosynthetic pathways involved in the formation of these unique substances.

Methods Employed: Sensitive bioassay methods, thin layer, column and gas chromatography have been used in the purification of the active principles, while NMR, mass spectroscopy, X-ray crystallography, microchemical techniques and radiochemical labeling are being utilized for structure elucidation. Isotopic tracer techniques have been used to study biosynthetic pathways.

Major Findings: The effects of batrachotoxin on electrogenic membranes have now been studied with a variety of biological preparations including the neuromuscular juncture (amphibian, mammalian and avian), giant axons (squid,

lobster), superior cervical ganglion of the intact mammalian heart, dog heart Purkinje fibers, amphibian skin and mammalian brain slices. In all systems the effects of batrachotoxin are explicable in terms of a selective and irreversible increase in permeability of membranes to sodium ions. This increase in permeability to sodium ions may be selectively and reversibly blocked by tetrodotoxin. Various analogs of batrachotoxin in which the pyrrole carboxylate moiety has been modified or has been replaced by a p-bromobenzoate moiety exhibit remarkable changes in relative activity towards pre- and post-synaptic structures and towards different biological preparations. The effects of certain analogs appear reversible.

Further investigations on the alkaloids from frogs of genus Dendrobates have led to the isolation and elucidation of the structure of a unique substance, histrionicotoxin A, found in extracts from Dendrobates histrionicus. This compound (7-(cis-1-buten-3-ynyl)-8-hydroxy-2(cis-2-penten-4-ynyl)-1-azaspiro[5.5]undecane) contains a spiro heterocyclic system unique in nature and the first example of acetylenic moieties in a compound of animal origin. The structure of a dihydro-derivative of histrionicotoxin has also been determined. In this dihydro derivative, one of the -HC=CH-C≡CH groupings has been replaced by a $-CH_2-\overset{H}{C}=C=CH_2$ grouping. This is the first example of such an allenic group in a natural product of animal origin. Cholesterol and related sterols and three other alkaloids were also isolated from skin extracts of the same frog, Dendrobates histrionicus.

Significance to Biomedical Research and the Program of the Institute: The exceptionally high toxicity of batrachotoxin, third among known toxins, its action on nerve preparations and its cardiotoxin properties gave the elucidation of its structure and correlations of structure with activity a high priority in biomedical research. Other compounds from frog extracts also have physiological activities which warrant their structural and pharmacological investigation. Examples of such compounds occurring in frogs and toads include the pumiliotoxins, histrionicotoxins, samandarine, the bufogenins, the catecholamines, indolealkylamines and histamines, and recently hypotensive polypeptides such as bradykinin and physalaemin. Because of its structural similarity to bufotenine and lysergic acid diethylamide, a thorough investigation of the properties of dehydrobufotenine is relevant to fundamental aspects of neurochemistry. The novel structure of histrionicotoxin related as it is to that of other alkaloids active in cholinergic mechanisms, warrants a thorough study both of its chemistry and pharmacology.

Proposed Course: The structures of various toxic alkaloids from frogs of genus Dendrobates will be elucidated. Further batrachotoxin will be purified in order to be able to provide qualified investigators with samples of this valuable research tool. The pharmacological activity of batrachotoxin analogs will be studied in detail. Radioactive batrachotoxin will be prepared and used to study its ultrastructural site of action. The unique oxidative cyclization of indolealkylamines to dehydrobufotenine will be investigated and attempts will be made to isolate the enzyme system responsible. The pharmacology of histrionicotoxin and derivatives will be investigated.

Honors and Awards: None

Publications:

Daly, J. W. and Witkop, B.: Chemistry and pharmacology of frog venoms. In Bücherl, W. and Buckley, E. (Eds.): Venomous Animals and Their Venoms (vol. II). New York, Academic Press, Inc., 1971, pp. 497-519.

Albuquerque, E. X., Daly, J. W., and Witkop, B.: Batrachotoxin, chemistry and pharmacology. Science, in press.

Daly, J. and Witkop, B.: Batrachotoxin, an extremely active cardio- and neurotoxin from the Colombian arrow poison frog, Phyllobates aurotaenia. Clin. Toxicol., in press.

Johnson, D. F. and Daly, J. W.: Biosynthesis of cholesterol and cholesterol acetate in Dendrobatid arrow poison frogs. Biochem. Pharmacol., in press.

Witkop, B.: Warum heute noch naturstoffchemie? Chemie in Unserer Zeit, in press.

Serial No. NIAMD-LC-28
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Photochemical Oxidation and Reduction of Diterpenes

Previous Serial Number: NIAMD-LC-19

Principal Investigator: J. A. Waters

Other Investigators: B. Witkop

Cooperating Units: I. Karle, U. S. Naval Research Laboratory, Washington, D. C.

Man Years:

Total: .3
Professional: .3
Others: 0

Project Description:

Objectives: To open up new synthetic routes for steroids and diterpenes of endocrinological or neurophysiological importance.

Methods Employed: The irradiations were conducted with a low-pressure mercury lamp and a high-pressure lamp in the presence of a photosensitizer.

Major Findings: Irradiation of methylkaur-15-en-19-oate (I) with a low-pressure ultraviolet lamp gave a crystalline oxirane derivative as the principal product. The structure of this compound is being elucidated by X-ray analysis. Ultraviolet irradiation of I with a high-pressure lamp under sensitizing conditions gave a dihydro derivative. Both reactions appear to be free radical in nature.

Significance to Biomedical Research and the Program of the Institute: These reactions assist the synthetic approaches to steroids and related diterpenes of cardiac, endocrinological and neurophysiological importance.

Proposed Course: To study in detail the mechanism of the photooxidation and photoreduction occurring in these reactions.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-29
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Anodic Decarboxylation of Glycidic Acids

Previous Serial Number: NONE

Principal Investigator: J. A. Waters

Other Investigator: B. Witkop

Cooperating Units: None

Man Years:

Total: .3
Professional: .3
Others: 0

Project Description:

Objectives: To study the effect of the Kolbe electrolysis on the α,β-epoxy group present in glycidic acids.

Methods Employed: Representative cycloalkyl, aromatic and steroidal glycidic acids were electrolyzed in methanol using smooth platinum electrodes.

Major Findings: Anodic oxidation of the steroidal glycidate derived from 5α-cholestan-3-one gave 3-acetyl-5α-cholest-2-ene (32%) and 3ξ-acetyl-3ξ-methoxy-5α-cholestane (11%). The cycloalkyl glycidates also gave α,β-unsaturated ketones and α-methoxy ketones.

Significance to Biomedical Research and the Program of the Institute: This method introduces a new synthesis of α,β-unsaturated ketones and α-methoxy ketones. These reactions assist the known synthetic routes to steroids of cardiac, endocrinological and neurophysiological importance.

Proposed Course: To study other carboxylic acids or amino acids of biological importance by the anodic oxidation method.

Honors and Awards: None

Publications:

Waters, J. A. and Witkop, B.: Anodic decarboxylation of glycidic acids. J. Org. Chem., in press.

Serial No. NIAMD-LC-30
1. Chemistry
2. Microanalytical Services and Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Section on Microanalytical Services and Instrumentation.

Previous Serial Number: 27

Principal Investigators: W. C. Alford and H. K. Miller

Other Investigators: Paul Parisius, Byron Baer, Alice Wong, William R. Landis, Benjamin Miller, Evelyn Peake, Anne Wright.

Cooperating Units: None

Man Years

Total: 9
Professional: 7
Other: 2

Project Description:

The Section on Microanalytical Services and Instrumentation is a service organization which provides instrumental and chemical analyses to approximately 130 research scientists at NIH and to a limited extent, to personnel of other government agencies including: Department of Agriculture, Food and Drug Administration, Naval Research Institute and U. S. Public Health Service hospitals. About 30 of the more common elements and five or more functional groups are routinely determined on a quantitative basis, using ultramicro, micro, and semi-micro techniques are required. The materials analyzed include organic and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and nonaqueous solvents and by mass spectrometry. Instrumental analyses include: mass spectrometry, infrared, nuclear magnetic resonance, ultraviolet, optical rotation, optical rotatory dispersion and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colorimetric,gasometric, coulometric, potentiometric and volumetric. Assistance in the interpretation of spectra is rendered on request.

During the past year the total number of analyses was about 13,000. These include: carbon-1500, hydrogen-1500, nitrogen-3200 (divided about 3:4:1 between Dumas,Kjeldahl and Nessler), phosphorus 200, sulfur-200, halogens-400, miscellaneous functional groups-100, metals-150, weight loss-650, miscellaneous-200, infrared spectra-1050, nuclear magnetic resonance spectra-2400, mass spectra-1500. In addition, several hundred instrumental analyses have been performed by individual research workers who use our equipment in the evening or weekends.

We have recently acwuired a Varian HA-100 Nuclear Magnetic Resonance Spectrometer. When its installation has been completed, we anticipate that the quality of n.m.r. spectra generated by the Section will be greatly enhanced.

A useful ancillary function of the Section is the provision of personnel (Mr. Benjamin Miller) to maintain an extensive stock of organic chemicals. This stockroom is for the use of NIAMD chemists primarily but is also open to scientists of other Institutes since there is no similar stock of organics elsewhere at NIH. Mr. Miller also maintains a stock of inorganic chemicals, glassware, compressed gases, shop tools and a small glass blowing facility. As time permits, he performs simple to moderately difficult glass blowing jobs for research personnel.

The Section Chief serves as Project Officer on Contract PH 43-65-32 between NIAMD and the Medical Research Council of England. This contract is for the partial support of a program which provides reference steroid compounds to qualified research workers in the United States as well as to a few foreign investigators. More than 1000 compounds are available and small amounts (1-5mg) are provided without cost for use as reference standards. Many of these materials are not available elsewhere in a comparable state of purity.

Honors and awards: None

Publications:

Landis, W. R., Perrine, T. D. and Thomas, George: A multiple column gas chromatograph. Journal of Chromatography, 53:125-134 (1970).

Landis, W. R., Alford, William C., Perrine, T. D., Thomas, George, M., Jr. and Anderson, Frank, O.: A slide value suitable for coupling as gas chromatograph to a mass spectrometer. Applied Spectroscopy, 25, No. 1, 44-46 (1971).

Cohen, L. A., Miller, H. K. and Peake, E. G.: Calculation of pKa values of alcohols from sigma constants and from the carbonyl frequencies of their esters. J. Org. Chem. In press.

Itano, H. A., Fogarty, W. M., Jr. and Alford, W. C.: The molar extinction coefficient of cyanmethemoglobin as determined by carbon analysis. Amer. J. of Clin. Path. 55, No. 2, 135-140, (1971).

Serial No. NIAMD-LC-31
1. Chemistry
2. Pharmacodynamics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Role of Cyclic Adenosine Monophosphate in the Central Nervous System.

Previous Serial Number: NIAMD-LC-29

Principal Investigator: John Daly

Other Investigators: M. Huang, J. Schultz

Cooperating Units: F. Bloom, St. Elizabeths Hospital, NIMH
E. Gruenstein, NIAMD:CE

Man Years

Total: 2.6
Professional: 1.3
Other: 1.3

Project Description:

Objectives: To determine the factors that govern the formation and metabolism of cyclic AMP in the central nervous system. To investigate the possible role of cyclic AMP in regulating biochemical reactions in brain tissue.

Methods Employed: Isotopically labeled compounds have been used to study the formation and breakdown of adenine nucleotides in brain slices and with partially purified enzymes.

Major Findings: Formation of cyclic AMP from a compartmentalized precursor pool of adenine nucleotides labeled by incubation with adenine-^{14}C is regulated in a synergistic manner by separate "receptors" for adenosine, histamine, and either norepinephrine or serotonin. The nature of the receptor sites has been investigated, revealing guinea pig cerebral cortex as the first tissue to be reported in which stimulated formation of cyclic AMP is largely mediated by a "β-receptor" for norepinephrine. A variety of antidepressants such as D-amphetamine, ritalin, pipradol, α-ethyltryptamine, harmaline and desipramine stimulate cyclic AMP formation in cerebral corical slices. Adenosine appears to stimulate cyclic AMP formation by both interaction with a regulatory "receptor" and

by incorporation into and thus enlargement of the precursor pool. Depolarizing agents, various metabolic inhibitors and certain adenosine analogs ultimately cause depletion of ATP in the precursor pool resulting in a blockade of cyclic AMP formation. Certain adenosine analogs such as 5-deoxyadenosine, however, appear to block cyclic AMP formation by direct inhibition of adenylcyclase. Simultaneous measurement of formation of cyclic AMP-^{14}C and of endogenous cyclic AMP revealed that the specific activity of cyclic AMP remains constant during stimulated formation in response to histamine, norepinephrine and ouabain.

Significance to Biomedical Research and the Program of the Institute: The key role of adenyl cyclase and cyclic AMP in regulating cellular activity in response to external stimuli in many biological systems make elucidation of their role in brain of fundamental importance to an understanding of function in this organ.

Proposed Course of Project: The morphological location of the labeled pools of adenine nucleotides will be determined. The role of phosphoribosyl transferase and adenosine kinase in maintaining this pool will be explored. The turnover of cyclic AMP formed after treatment with amines, adenosine or depolarizing agents will be measured. Possible secondary effects of elevated cyclic AMP levels in brain tissue will be sought. The *in vivo* effects of drugs on the responsiveness of the cyclic AMP system will be explored. The effects of electrical pulsation, prostaglandins and thyroxine on cyclic AMP levels will be probed. The mechanism whereby antidepressants elevate cyclic AMP levels will be determined. Interactions between amines, adenosine and depolarizing agents will be further investigated. Attempts to label the precursor pool with adenine analogs will be made.

Honors and awards: None

Publications:

Huang, M., Shimizu, H. and Daly, J. W.: Regulation of Adenosine Cyclic 3',5'-Phosphate Formation in Cerebral Cortical Slices: Interactions Among Norepinephrine, Histamine and Serotonin. Mol. Pharmacol. 7:155 (1971).

Shimizu, H. and Daly, J.: Effect of Depolarizing Agents on Accumulation of Cyclic Adenosine 3',5'-Monophosphate in Cerebral Cortical Slices. Europ. J. Pharmacol. In press.

Shimizu, H., and Daly, J. W.: Methods for the Measurement of Cyclic AMP in Brain. Methods and Techniques of Neuroscience. In press.

Shimizu, H., and Daly, J.: Formation of Cyclic Adenosine 3',5'-Monophosphate from Adenosine in Brain Slices. Biochim. Biophys. Acta. 22:465 (1970).

Serial No. NIAMD-LC-32
1. Chemistry
2. Pharmacodynamics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Pharmacodynamic Amines and Enzymes Involved in Their Metabolism.

Previous Serial Number: NIAMD-LC-28

Principal Investigator: C. R. Creveling

Other Investigators: J. Daly, D. Jerina, N. Kaubisch, S. Milstien, W. Lutz.

Cooperating Units: J. Milstien, NIAMD:LPB
W. Lovenberg, NHI:ET
G. W. A. Milne, NHI:LC

Man Years

Total: 3.2
Professional: 1.9
Other: 1.3

Project Description:

Objectives: To study the basic chemistry and biochemistry of the catecholamines, phenolic amines, indolealkylamines, imidazolealkylamines and their precursors and transformation products. To develop new techniques for the investigation of transport metabolism and binding phenomena as related to these amines and their interactions with various drugs. To isolate and purify enzymes operative in the biosynthesis and metabolism of biogenic amines. To determine the physical properties of these enzymes and the effect of pH, temperature, substrate specificity, reversible and irreversible inhibitors and the ionic composition of the incubation media. To develop assays and to apply these enzymatic assays to determine specific biogenic amines and to study the distribution and variation of enzyme levels.

Methods Employed: Drugs and substrates labeled with tritium, deuterium or C^{14} have been used to study the metabolic fate of endogenous amines and amino acids and the underlying mechanisms of uptake, release and metabolism of these compounds. Standard methods of enzyme purification have been used including: differential centrifugation, density gradient centrifugation, gel filtration,

electrophoresis, standard column chromatography and column chromatography using cellulose specifically charged with substrates. New synthetic techniques for synthesis of amines and amino acids have been developed.

Major Findings: Catechol-O-methyltransferase (COMT) purified 400 fold from rat liver contains two enzymatically active glycoproteins with molecular weights of 25,000-31,000 and 42,000-54,000. Substituted catechols are converted to two isomeric O-methylated products with COMT and S-adenosylmethionine. The meta/para ratios are dependent on the nature of the ring substituent, the pH and the concentration of the divalent cation (Mg^{++}, Mn^{++} or Zn^{++}). Contrary to early reports, COMT is inhibited rather than activated by Co^{++}, Cd^{++}, Fe^{++} and Ni^{++}. The enzyme is fully activated towards ionized catechols by trace amounts of tightly bound undialysable Mg^{++}, while for unionized substrates full activation requires large amounts of additional Mg^{++}. Electron paramagnetic resonance studies with Mn^{++} and purified COMT indicate that 3-4 Mn^{++} are bound to the enzyme with a binding constant of 1.5×10^{-4}M. S-adenosylamethionine,but not substrate increases the affinity of COMT for Mn^{++}. In both cases the number of metal ion sites does not change. Conversely, divalent metal ions decrease the affinity of COMT for S-adenosyl-methionine and slightly increase the affinity for substrate. The affinity of S-adenosyl-homocysteine, a product of the reaction and a competitive inhibitor with respect to S-adenosylmethionine, is unaffected by metal ion concentration.

The usual hyperbolic relationship between reaction rate and substrate concentration was observed with COMT and many catechols. However, with norepinephrine, epinephrine, dopamine and isoproterenol, a sigmoid curve pertained, suggesting that COMT is an allosteric protein whose activity can be enhanced by these physiological substrates at concentrations greater than 10^{-4}M.

The 3-thio analogs of dopamine, dopa and norepinephrine were synthesized. The 3-thio-4-hydroxyphenethylamine was a potent irreversible inhibitor of COMT *in vitro*. The inhibition could be reversed with dithiothreitol. The compound was sympathomimetic *in vivo*.

Chemical ionization mass spectrometry of dansylated amines from biological sources promises to provide a means of identifying such derivatives in crude mixtures since the method affords only two mass spectral peaks, corresponding to the parent ion and to a fragment common to all dansylated amines.

A simple readiometric assay for tryptophan hydroxylase has been developed using 5-tritiotryptophan prepared by reductive tritiation of 5-bromotryptophan with tritium gas of moderate specific activity. This procedure avoids radiolytic random labeling of the

tryptophan ring system.

The 4-O-methyl derivative of dopa has been synthesized for studies on its metabolism and pharmacological activity.

Significance to Biomedical Research and the Program of the Institute: The biosynthesis and metabolism of biogenic amines are of fundamental importance to the fields of neurochemistry, pharmacology and have a direct bearing on the understanding of the functioning of the cardiovascular system.

Proposed Course of Project: The binding and release of norepinephrine as influenced by drugs will be explored further, as will interactions of certain amines and drugs at storage and receptor sites. All possible trihydroxyphenethylamines will be synthesized and studied since the two known isomers 3,4,5-trihydroxyphenethylamine and 6-hydroxydopamine exhibit remarkable activities will respect to interference with norepinephrine storage. The mode of action and selectivity of 6-hydroxydopa in eliciting depletion of catecholamines in the central nervous system will be studied. Studies on active site labeling and the primary structure of catechol-O-methyltransferase will be undertaken. Studies on inhibitors of tyrosine hydroxylase, phenylalanine hydroxylase, dopamine-β-hydroxylase and catechol-O-methyltransferase will be continued. Attempts will be made to prepare antibodies to catechol-O-methyltransferase. The possible significance of false transmitters for histamine and serotonin will be explored. An attempt to rationalize the kinetic interactions of COMT, substrate, metal ion and S-adenosylmethionine in terms of a reaction mechanism is in progress. The interactions of thio analogs of phenolic and catecholic biogenic amines with a variety of enzymes and biological systems will be explored in detail.

Honors and awards: None

Publications:

Creveling, C. R., Dalgard, N., Shimizu, H. and Daly, J. W.: Catechol-O-methyltransferase III. Meta-and para-O-Methylation of Catechol Amines and Their Metabolites. Mol. Pharmacol. 6:691 (1970).

Lovenberg, W., Bensinger, R. E., Jackson, R. L. and Daly, J. W.: Rapid Analysis of Tryptophan in Rat Tissue Using 5-^{3}H-Tryptophan. Anal. Biochem. In press.

Serial No. NIAMD-LC-33
1. Chemistry
2. Pharmacodynamics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Mechanism of Enzymatic Hydroxylation and The Role of Reactive Intermediates in Drug Metabolism.

Previous Serial Number: NIAMD-LC-31

Principal Investigator: Donald M. Jerina

Other Investigators: J. Daly, N. Kaubisch, A. Jeffrey, F. Oesch, and B. Witkop.

Cooperating Units: D. Boyd, Univ. Belfast, Belfast, Ireland
H. Ziffer, NIAMD:LPB
D. Reed, Oregon State Univ., Corvallis, Oregon
O. Chapman, Iowa State Univ., Ames, Iowa
J. Rice, NCI:ET

Man Years

Total: 2.9
Professional: 2.6
Other: 0.3

Project Description:

Objectives: To study the basic mechanism of oxygen activation and enzymatic incorporation into organ substrates. To explore enzymatic and non-enzymatic reactions of labile intermediates such as epoxides and cyclic peroxides.

Methods Employed: Isotopically labeled substrates, water and oxygen gas, have been used to study the oxidation of organic compounds with a variety of enzymes and the subsequent metabolism of labile intermediates. Nuclear magnetic resonance spectroscopy, mass spectrometry, x-ray crystallography, and other physico-chemical techniques have been employed frequently in these studies.

Major Findings: Comparison of activities of a variety of epoxide substrates with microsomal epoxide hydrase and a soluble purified preparation of epoxide hydrase gave purification factors of 26-30 for 8 of these substrates. With benzene oxide as substrate however, a purification of only 4 was obtained indicating the pres-

ence of at least one homologous epoxide hydrase in microsomes which has been largely lost during purification.

The activity of epoxides as substrates or inhibitors of epoxide hydrase is strongly dependent in the nature, number and stereochemistry of substituents of the epoxide (oxirane) ring. Inhibition was measured with styrene-^{3}H oxide as substrate. 1-Substituted oxiranes with a large hydrophobic substituent are good substrates and competitively inhibit hydration of styrene oxide. 1,1-Disubstituted and cis-1,2-disubstituted oxiranes are less active while trans-1,2-disubstituted, tri- and tetra-substituted oxiranes are virtually inactive. Certain alicyclic oxiranes are poor substrates but are excellent noncompetitive inhibitors. The most potent epoxide inhibitor, 1,1,1-trichloropropene-2,3-oxide is a poor substrate. It causes a rather rare type of inhibition, i.e., uncompetitive with respect to substrate. It is virtually inactive as an inhibitor in vivo. Epoxide hydrase may be activated by certain compounds such as alcohols. The most active of such compounds is metyrapone, which is, however, inactive in vivo. Radiometric assays for epoxide hydrase and aryl hydroxylases have been modified to increase their sensitivity. Both activities are found in adult but not fetal rat skin. Only the hydrase activity was detected in skin tissue cultures.

To further explore alkyl migration during aryl hydroxylation and arene oxide formation, 1-methylnaphthalene oxide, and 1,2-dimethylnaphthalene oxide were prepared. The latter compound rearranged with methyl migration to 2,2-dimethyl-2,3-dehydro-1-tetralone.

Cis-1,2-Dihydroxy-1,2-dihydro naphthalene was identified as the sole dihydrodiol product from naphthalene metabolism in a variety of bacteria, thereby correcting earlier speculation on bacterial formation of trans-dihydrodiols. Preliminary attempts to implicate cyclic peroxides or perepoxides as intermediates with azide as a trapping agent were unsuccessful.

The reaction of benzene-3,6-^{2}H oxide with azide, methyl lithium and disulfide was examined. Methyl lithium added abnormally to yield cis-1-hydroxy-2-methyl-1,2-dihydrobenzene-2,5-^{2}H, while the other agents added normally to yield trans-1-hydroxy-2-substituted-1,2-dihydrobenzene-3,6-^{2}H.

A model hydroxylating system utilizing ferrous ions, O_2 and thiosalicylic acid was shown to form both cis- and trans-1,2-dihydro-1,2-dihydroxynaphthalene, indicating that this may be a useful model for investigating activation of oxygen in both mono- and dioxygenase-catalyzed reactions.

Fungal metabolism of aromatic substrates occurs by aryl hydroxylation, O-demethylation and benzylic oxidation. Aryl hydroxylation in most instances is accompanied by an NIH Shift comparable to that seen in mammalian systems.

The low temperature photolysis of benzene oxide, naphthalene oxide and phenanthrene oxide yields the keto forms of phenol, naphthol and phenanthrol providing evidence that such compounds can exist as transient intermediates during rearrangement of biologically produced arene oxides. Keteons, epoxide shifts, and from phenanthrene oxide, a novel oxepine were also detected after photolysis of those arene oxides.

A variety of approaches to the synthesis of the cyclic peroxides, postulated as intermediates in microbial metabolism of aromatic rings, have been explored. As yet none have been successful in terms of formation of the ultimate arene cycloperoxide. Autoxidation of 1,4-dihydronapthalene surprisingly gave 2-hydroperoxy-1,2-dihydronaphthalene (singlet oxygen gave no reaction). This hydroperoxide rearranged to the naphtho-oxepine and could be reduced to 3-hydroxy compound (a hydrate of napthalene). The latter compound also forms from 1,4-dihydronaphthalene-2-3 oxide with base.

Significance to Biomedical Research and the Program of the Institute: Enzymatic oxidation reactions are extremely important in many biosynthetic pathways and in the metabolism, detoxication and action of most drugs.

Proposed Course of Project: The mechanism(s) of enzymatic hydroxylation will be further investigated. Arene epoxides of other aromatic substrates will be synthesized and attempts will be made, using tracer technology, to demonstrate their intermediacy in aryl oxidation. Mechanism of action of epoxide hydrase and its role in drug detoxification will be studied. Assay for "benzene oxide hydrase"will be developed and this enzyme will be investigated. The effect of various parameters on the stereochemical opening of epoxides with the microsomal epoxide hydrases will be explored. Further studies on model systems including photolysis of N-oxides, and Fe^{++}, O_2, thiosalicylic acid will be conducted. Isotope effects on aryl hydroxylases with a variety of substrates may provide a criterion for intermediacy of arene oxides in phenol formation.

Honors and awards: None

Publications:

Oesch, F., Jerina, D. M. and Daly, J. W.: Substrate Specificity of Hepatic Epoxide Hydrase in Microsomes and in a Purified Preparation: Evidence for Homologous Enzymes. Arch. Biochem. Biophys. In press.

Oesch, F., Jerina, D. M., and Daly, J.: A Radiometric Assay for Hepatic Epoxide Hydrase Activity with Styrene-7-^{3}H Oxide. Biochim. Biophys. Acta. 227:685 (1971).

Oesch, F. and Daly, J.: Solubilization, Purification and Properties of a Hepatic Epoxide Hydrase. Biochim. Biophys. Acta. 227:692 (1971).

Jerina, D. M., Daly, J. W. and Witkop, B.: Migration of Substituents During Hydroxylation of Aromatic Substrates (NIH Shift). Oxidations with peroxytrifluoracetic acid. Biochemistry. 10:366 (1971).

Daly, J.: Metabolism of Acetanilides and Anisoles with Rat Liver Microsomes. Biochem. Pharmacol. 19:2979 (1970).

Jerina, D. M., Daly, J. W., Jeffrey, A. M., and Gibson, D. T.: Cis-1,2-dihydroxyl-1,2-dihydronaphthalene: A Bacterial Metabolite from Naphthalene. Arch. Biochem. Biophys. 142:394 (1971).

Serial No. NIAMD-LC-34
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Study of Solanocapsine and its Derivatives

Previous Serial No.: None

Principal Investigator: Yoshio Sato

Other Investigators: None

Cooperating Units: Dr. Yao Teh Chang
NIAMD, LBP
Bethesda, Md.

Professor F. G. Standaert
Department of Pharmacology
Georgetown University
Washington, D. C.

Man Years

Total: 0.45
Professional: 0.2
Other: 0.25

Project Description:

Objective: To prepare various derivatives of solanocapsine for pharmacological activity (antileprosy and neuromuscular blocking effect).

Methods Employed and Major Findings: It has been found that solanocapsine hydriodide possesses considerable activity against Mycobasterium Lepraemurium (mouse leprosy). Various derivatives of solanocapsine are being prepared to enhance activity and lessen its toxicity (testing by Dr. Chang).

The bis quaternary methiodide of solanocapsine has also been found to exhibit some neuromuscular blocking activity. Modification of the molecular structure is being made to enhance activity and eliminate the undesirable factors. (Testing by Prof. Standaert)

Significance to Biomedical Research and the Program of the Institute: Since the leprosy bacteria has developed some tolerance towards current drugs in use, new chemotherapeutics are in demand. The need for rapidly acting non-depolarizing myoneural blocking agent of short duration without undesirable side effects in anaesthesia is recognized.

Proposed Course of Project: Various modifications of the molecular structure of solanocapsine will be attempted to ascertain the usefulness of the compound.

Honors and Awards: None

Publications:

Nagai, M. and Sato, Y.: Structure of Solanocapsine, Synthesis of 3-acetoxy-22,26-acetylepimino-16α,23-epoxy-5α,22αH,25βH-cholestan-23β-ol. Tetrahedron Letters 33: 2911-2913, 1970.

Serial No. NIAMD-LC-35
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Steroidal Alkaloids - Reactions of Solasodine

Previous Serial No.: NIAMD-LC-32

Principal Investigator: Masahiro Nagai

Other Investigator: Yoshio Sato

Cooperating Units: None

Man Years

Total: 0.95
Professional: 0.7
Other: 0.25

Project Description:

Objective: To exploit and extend the basic chemical knowledge of solasodine for utilization as starting material for the production of biologically active steroid hormones.

Methods Employed and Major Findings: The reaction of solasodine-3-acetate with phosgene, followed by aq. dimethylamine treatment is of some interest. In the presence of pyridine as a catalyst, 3β-acetoxy-22,26-epimino-$\Delta^{5,22}$-cholestadiene-16β,26(N)-urethane (1) is obtained in a good yield along with a small amount of 26-N',N'-dimethylcarbamido-3β-acetoxy-$\Delta^{5,20(22)}$-furostadiene (2). On the other hand in the presence of triethylamine in lieu of pyridine the reaction proceeds to yield 26-N',N'-dimethylcarbamido-3β-acetoxy-$\Delta^{5,22}$-furostadiene (3), N-(N',N'-dimethylaminoformyl)solasodine-3-acetate (4), 26-N',N'-dimethylcarbamido-3β-acetoxy-Δ^{5}-furosten-22-ol (5) and small amounts of (1) and (2). Upon treatment with acetic acid, compounds (3), (4) and (5) yield (2) in a quantitative manner. Chromic acid oxidation followed by acetic acid hydrolysis of (2) affords 3β-acetoxy-$\Delta^{5,16}$-pregnadiene-20-one, a well known intermediate for the synthesis of many biologically active steroid hormones.

Significance to Biomedical Research and the Program of the Institute: With increase in demand for oral contraceptives, there is renewed interest in the steroidal alkaloid, solasodine, as starting material for production of steroid hormones. The method described above for the degradation of solasodine to pregnadienolone acetate may have some industrial significance.

Proposed Course of Project: A search for an economical source and effective procedure for conversion of natural products into biologically active steroid hormones will continue.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-36
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Reports
July 1, 1970 through June 30, 1971

Project Title: The Synthesis of Solacongestidine and Solafloridine

Previous Serial No.: Same

Principal Investigator: Masahiro Nagai

Other Investigators: Yoshio Sato, Genjiro Kusano

Cooperating Units: None

Man Years

Total: 0.58
Professional: 0.58
Other: 0

Project Description:

Objective: A number of hypotheses on the biosynthesis of solanum alkaloids have been advanced with very little experimental evidence. In order to obtain sufficient quantities of the alkaloid and at the same time to understand the biogenetic origin of the solanum alkaloids in plants, a biochemically oriented approach to the conversion of kryptogenin to Δ^5-solacongestidine is now planned.

Method Employed and Major Findings: Although solasodine has now been successfully converted into solacongestidine and solafloridine, the yields are rather small. A more biogenetically feasible approach starting with kryptogenin, a steroidal sapogenin, proceeds as follows: Kryptogenin diacetate is converted into the 16-thioketal derivative and desulfurized with Raney nickel. Alkaline saponification of the resulting 16-deoxo product affords cholest-5-ene-3β,26-diol-22-one(1). Partial tosylation of the latter yields the 26-tosylate which when reacted with iodide ion leads to 3β-hydroxy-26-iodo-5-cholesten-22-one. It is hoped that the amination of the iodo compound will be attended with concurrent cyclization to afford the desired steroidal alkaloid.

Significance to Biomedical Research and the Program of the Institute: If the partial synthesis from kryptogenin is successful in the above manner, the steroid sapogenin or some modified form may be viewed as a possible precursor in the biosynthesis of steroidal alkaloids.

Proposed Course of Project: In the event that Δ^5-solacongestidine is synthesized from 16-deoxokryptogenin (1) it will be labelled with radio isotopes and fed into solanum plants for the study of steroidal alkaloid biosynthesis.

Honors and Awards: None

Publications:

Kusano, G., Aimi, N., and Sato, Y.: Conversion of Solasodine to Solafloridine and Dihydrosolacongestidine Acetate. J. Org. Chem. 35: 2624-2626, 1970.

Serial No. NIAMD-LC-37
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Study of the Constituents of Solanum Xanthocarpum

Previous Serial No.: None

Principal Investigator: Genjiro Kusano

Other Investigator: Yoshio Sato

Cooperating Unit: Malcolm J. Thompson and William E. Robbins
Insect Physiology Laboratory
Agricultural Research Service
U. S. Department of Agriculture
Beltsville, Maryland 20705

Man Years

Total: 0.53
Professional: 0.53
Other: 0

Project Description:

Objective: To isolate and identify the highly toxic component toward the housefly larvae present in the extracts of S. xanthocarpum.

Methods Employed and Major Findings: A systematic chromatographic separation of the alcoholic extacts from S. xanthocarpum has resulted in the separation and identification of the following compounds: solasonine, solamargine, solasodine, Δ^7-citrostanol-3-benzoate, Δ^7-citrostandiol(3β,22), Δ^7-citrostandiol(3β,22)-3-benzoate, Δ^7-citrostanol-3-O-glycoside, carpesterol debenzoxycarpesterol, cycloartanol, cycloartenol, β-sitosterol, stigmasterol and campesterol. Although the three steroidal alkaloids have some larvaecidal activity, the most toxic substance still eludes our grasp.

Significance to Biomedical Research and the Program of the Institute: The isolation, identification and synthesis of a potent insecticide, yet harmless to man and animals, for the control of the common household fly would be of considerable significance in the field of health and general sanitation. Furthermore, the isolation and identification of a number of steroid and triterpene intermediates adds to our knowledge concerning the biogenesis of plant steroids.

Proposed Course of Project: A further exhaustive study will be made to ascertain the identity of the toxic substance. Structure activity studies will be made in the event the compound shows promise.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-38
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Synthesis of Solaphyllidine

Previous Serial No.: NIAMD-LC-33

Principal Investigator: Genjiro Kusano

Other Investigator: Yoshio Sato

Cooperating Unit: None

Man Years

Total: 0.53
Professional: 0.53
Other: 0

Project Description:

Objective: To synthesize solaphyllidine (22,26-epimino-5α-cholestan-4-one-3β,16α,23-triol 16-monoacetate), a new steroidal alkaloid isolated from Solanum hypomalacophyllum Bitter, utilizing Δ^5-solafloridine as starting material.

Methods Employed and Major Findings: Δ^5-Solafloridine prepared synthetically according to modified methods adopted in the synthesis of solafloridine (see annual report No. NIAMD-LC-35) was transformed into the 3,16-diacetyl derivative, oxidized to the 23-oxo-compound, reduced to the carbinol amine, 4-deoxo-Δ^5-solaphyllidine with $NaBH_4$. This compound was hydrogenated to 4-deoxo-solaphyllidine and found to be identical with the 4-deoxo compound obtained from solaphyllidine. Some attempts will be made to convert 4-deoxo-Δ^5-solaphyllidine to solaphyllidine.

Significance to Biomedical Research and the Program of the Institute: The structure of solaphyllidine possesses features for consideration as a new intermediate in the biosynthesis of Solanum and Veratrum alkaloids. Its synthesis therefore is of considerable academic interest. Since these alkaloids in general possess some pharmacological activity, the synthesis will permit large scale screening for biological activity.

Proposed Course of Project: If the compound shows some physiological activity, the study will be continued.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-39
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: (1) The Synthesis of Solanocapsine
(2) Conversion of Solaphyllidine to Solanocapsine
(3) Steroidal Alkaloids - Isomerism of Solasodine Formates
(4) The Study of the Alkaloids from Solanum congestiflorum (Natri)

Previous Serial No.: (1) NIAMD-LC-31
(2) NIAMD-LC-34
(3) NIAMD-LC-37
(4) NIAMD-LC-38

Principal Investigator: (1) Masahiro Nagai
(2) Genjiro Kusano
(3) Genjiro Kusano
(4) Genjiro Kusano

Other Investigator: (1) Yoshio Sato
(2) Yoshio Sato
(3) Yoshio Sato
(4) Yoshio Sato

Cooperating Units: (1) None
(2) Professor Alfredo Usubillaga
Instituto de Investigacion Quimica
Universidad de Los Andes
Facultad de Farmacia
Merida, Venezuela
(3) Edwin D. Becker, NIAMD-LPB
(4) None

(1) This project has been completed. (2) This project is being held in abeyance. (3) This project is being held in abeyance. (4) This project has been discontinued.

Serial No. NIAMD-LC-40
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Metabolism of C^{14}-Labelled Steroids by Adrenals from Pseudohermaphrodite Rats

Previous Serial No.: NIAMD-LC-43

Principal Investigator: David F. Johnson

Other Investigator: Nancy Lamontagne

Cooperating Unit: C. Wayne Bardin, Formerly Endocrinology, NCI
Present: Pennsylvania State Medical School
Hershey, Pennsylvania

Man Years

Total: 1.25
Professional: 1
Other: 0.25

Project Description:

Objective: The above mentioned cooperating unit has been studying a unique colony of rats in which a genetic defect, carried by normal females, is passed on to half the genetic males. Half the genetic males are pseudohermaphrodites. These pseudos have both male and female characteristics, although the testes are internal. It has been demonstrated that they have a defect of testosterone synthesis, suggesting a general deficiency of the 17-β-hydroxylase enzyme systems may be involved. The present study is designed to test this hypothesis by investigating the metabolism of progesterone in the adrenal glands of these animals.

Methods Employed and Major Findings: Radioactive progesterone was incubated with normal rat adrenals (male and female) and adrenals from pseudohermaphrodite rats. Extracts of the homogenized adrenal tissue were investigated by thin-layer chromatography and scanning for radioactivity. Comparisons with known standards were made for identification. Extracts of the incubation media were chromatographed on silicic acid columns by gradient elution techniques. Individual fractions were first isolated by plotting U.V. absorption of known carrier standards and radioactivity versus tube number. Isolated zones were then characterized by thin-layer and radioactive scanning.

Analysis of the tissue, after separation of the aqueous incubation media, revealed no significant difference in the products of progesterone metabolism when normal male and female were compared to the pseudo. The major metabolites identified by TLC were unchanged progesterone, corticosterone, deoxycorticosterone and 18-hydroxydeoxycorticosterone.

Extracts of the incubation media were analyzed by both column chromatography and TLC. In addition to unchanged progesterone; 11-keto-P,4-androslene-3,17-dione,11β-OH-androstenedione, and 11α-OH-P were tentatively identified. The amount of unmetabolized progesterone was significantly higher for the pseudo rat than for the male and similar to that found for the female. With the exception of 11β-OH-androstenedione, the other metabolites were also produced in higher amounts for the pseudo than the male and even higher than that for the female. Of particular significance was the production of 4-androstene-3,17-dione, a direct precursor of testosterone synthesis. Although no testosterone could be detected in the adrenal tissue incubation of male, female, or pseudo, these findings are similar to those of Bardin in a study of testicular tissue function. He found direct evidence of decreased 17β-hydroxydehydrogenase activity as reflected in decreased testosterone synthesis. Our indirect evidence is indicative of a similar lack in adrenal tissue of these animals.

Significance to Biomedical Research and the Program of the Institute: The preliminary findings of the present study suggest further experiments which may lead to a better understanding of the interrelated biosynthesis of adrenocortical steroids, androgens, and estrogens. These animals are especially suited to such study since the defective testosterone synthesis has been well established in testicular tissue and our present findings are strongly indicative of a defective 17-β-hydroxysteroid dehydrogenase enzyme system in the adrenal tissue.

Proposed Course of Project: A more detailed study of the biosynthetic pathways in the adrenal gland of the pseudohermaphrodite rat is planned. The proposed work would involve comparisons with normal male and female adrenals on both a quantitative and kinetic basis.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-41
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: In Vitro and In Vivo Biosynthesis of Frog Toxins

Previous Serial No.: NIAMD-LC-39

Principal Investigator: David F. Johnson

Other Investigator: John W. Daly

Cooperating Units: None

This project has been terminated. A publication entitled "Biosynthesis of Cholesterol and Cholesterol Acetate in Dendrobatid Arrow-Poison Frogs" by David F. Johnson and John W. Daly has been accepted for publication in Biochemical Pharmacology.

Serial No. NIAMD-LC-42
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Toxicity Studies of Solanum Bulbocastanum

Previous Serial No.: NIAMD-LC-40

Principal Investigator: David F. Johnson

Other Investigator: Yoshio Sato

Cooperating Unit: Potato Introduction Project
USDA Agricultural Research Service
Department of Horticulture
University of Wisconsin
Madison, Wisconsin

This project held in abeyance pending repeat of isolation and testing if plants are available in the next growing season under ideal conditions of growth.

Publication:

Johnson, D. F. and Sato, Y.: Sterols from Solanum Polyadenium and solanum bulbocastanum. Advancing Frontiers of Plant Sciences 23: 17-24, 1968.

Serial No. NIAMD-LC-43
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biosynthesis of Stigmasten-3β-ol and 3',5'-Cyclic AMP by the Slime Mold (Dictyostelium discoideum)

Previous Serial No.: NIAMD-LC-41

Principal Investigator: David F. Johnson

Other Investigators: None

Cooperating Unit: Micah Krichensky, D, IR

This project being held in abeyance, awaiting contribution and write-up of the cooperating unit.

Serial No. NIAMD-LC-44
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Column Chromatography of Adrenocorticol Steroids and Ketosteroids by Gradient Elution

Previous Serial No.: NIAMD-LC-42

Principal Investigator: David F. Johnson

Other Investigator: Nancy Lamontagne

Cooperating Unit: Grant C. Riggle, Instrument Engineering and Development Branch, Division of Research Services, NIH

Man Years

Total: 1.0
Professional: 1.0
Other: 0

Project Description:

Objective: The demands of chromatographic resolution in complex mixtures of adrenal steroids require precise elution gradients that can be varied at will and maintained within precise limits of solvent concentration. Such a system was developed with the cooperating unit. Testing the utility of the gradient system involves application of the gradient methodology to model steroid mixtures and mixtures from biological sources. An integral part of the chromatographic process involved development of analytical parameters of column preparation, using water impregnated silicic acid, and gradient design to effect separation of selected adrenal and ketosteroids. Changes in the method of commercial preparation of silicic acid formerly used in this laboratory, resulting in drastic alteration of its properties, necessitated an extensive survey of commercially available material.

Methods Employed: The study of chromatographic properties of commercial silicic acid required a survey of a number of available samples and a determination of the effect that activation and water load had on their separating ability with a standard mixture.

Using one of the commercially available silicic acids, the conditions of activation and water load were varied in an investigation of optimal conditions for qualitative separation and recovery of a model mixture of

adrenal and ketosteroids. As part of this study, various elution gradients were used in the development of a standard method of analysis.

Major Findings: The survey of commercial silicic acids revealed a wide variation in properties as a supporting phase for partition chromatography of steroids. A number of preparations were unusable, regardless of the alteration of liquid stationary phase. One of the products proved to be most suitable for the purpose, and analytical parameters were determined and adopted as a routine preparation for our method.

Using the punched-tape gradient system, a standard method was developed for the analysis of adrenocortical and ketosteroids in a mixture on a single column. The method has been successfully applied to both model mixtures and those resulting from incubation experiments using adrenal tissue and radioactive steroid precursors.

Significance to Biomedical Research and the Program of the Institute: Investigations of the biosynthetic pathways involved in adrenal metabolism using radioactive precursors require precise chromatographic separation of metabolites. The present method allows for determination of adrenal and ketosteroids on a single column, facilitating interpretation of biosynthetic pathways involved in adrenal metabolism.

Proposed Course of Project: The method will be used in conjunction with thin-layer techniques in the analysis of a variety of mixtures resulting from incubation of adrenal tissue with radioactive steroid precursors.

Honors and Awards: None

Publications:

Johnson, D. F. Lamontagne, N. S., Riggle, G. C., and Anderson, F. O. A tape controlled gradient elution chromatography system. Proceedings of the 23rd Annual Conference on Engineering in Medicine and Biology 12: 337, 1970.

Lamontagne, N. S. and Johnson, D. F.: Chromatography of adrenocortical steroids on silicic acid columns. J. Chromatography 53: 225-232, 1970.

Lamontagne, N. S. and Johnson, D. F.: Determination of 17-ketosteroids and adrenocortical steroids by gradient elution chromatography on a silicic acid column. Steroids 17: 365-375, 1971.

Serial No. NIAMD-LC-45
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Study of the Photochemical Reactions of Certain Derivatives of Indole and Related Heterocyclic and Carbocyclic Compounds.

Previous Serial No.: NIAMD-LC-44

Principal Investigator: Calvin M. Foltz

Other Investigators: None

Cooperating Units: None

Man Years

Total: 1.0
Professional: 1.0
Other: 0

Project Description:

Objective: To develop organic chemical and photochemical methods of potential utility in the study of biological and medical problems and to synthesize organic compounds of biological and medical interest. To study the mechanism of the photocyclodehydrohalogenation reaction produced by uv irradiation of N-chloroacetyltryptamine and related compounds and to study the scope of its applicability to other heterocyclic and non-heterocyclic systems. To investigate other photodehydrohalogenation reactions and to apply and further develop the phototitration technique in such studies. To study the scope and mechanism of the rearrangement which has been found to take place on uv irradiation of 3-(3'-chloro-2'-oxopropyl)indoline-2-one.

Methods Employed: Standard organic chemical techniques; irradiation of solutions of organic compounds with ultraviolet radiation generated by mercury-vapor lamps; column, gas, and thin layer chromatography; and ultraviolet, visible, infrared, nuclear magnetic resonance, and mass spectroscopy.

Major Findings: Photolysis of solutions of N-chloroacetyltryptamine, N-chloroacetyl-3-(3'-aminopropyl)indole and N-chloroacetyl-3-(4'-aminobutyl)-indole in tetrahydrofuran or aqueous methanol with the radiation from a high pressure or a low pressure mercury-vapor lamp results in cyclization at the 4-position of the indole moiety to produce tricyclic lactams containing eight-, nine- and ten-membered amide rings respectively. The respective products and approximate yields are the following: 6-oxo-3,4,6,7-tetrahydro-

1H,5H-azocine[4,5,6-cd]indole(50%), 7-oxo-3,4,5,6,7,8-hexahydro-1H-azonin [6,5,4-cd]indole(30%), and 8-oxo-3,4,5,6,8,9-hexahydro-1H,7H-azecin[6,5,4-cd]indole(20%). This phase of the study of this problem has nearly been completed. These photocyclodehydrohalogenation reactions are moderately fast and automatic titration of the hydrogen ion liberated in the course of the reaction has been a convenient method for following the course of the reactions. This technique is being developed for the study of the kinetics and mechanism of these and related reactions and as an aid in extending the raction to other systems.

The reaction has been applied to N-chloroacetyl-2-(α-naphthyl)ethylamine (1) as a prototype of aromatic polycyclic systems. Irradiation of compound 1 in MeOH-HoH (1:1) with a high pressure mercury-vapor lamp resulted in loss of HCl and the formation of the tricyclic lactam (2), 2-oxo-2,3,4,5-tetrahydro-1H-naphth[1,8-de]azocine, in an isolated yield of 47%. Compound 2 was reduced to the amine (3), 2,3,4,5-tetrahydro-1H-naphth[1,8-de] azocine, with diborane. Compound (3) was acetylated to obtain the amide (4), N-acetyl-2,3,4,5-tetrahydro-1H-naphth[1,8-de]azocine. Compounds 2, 3, and 4 were characterized by combustion analysis and uv, ir, nmr, and mass spectroscopy. Some bands of the nmr spectra of 2, 3, and 4 display a temperature dependence which confirmed the correctness of the structural assignments. Additional study of this temperature dependence and that of related polycyclic lactams produced in the course of this work is contemplated. Phototitrations of 1 under various conditions indicated that the reaction occurs at about the same rate as that of N-chloroacetyltryptamine. On the basis of phototitrations under various conditions a working mechanism has been proposed for the reaction.

The 3-carbonyl function of isatin, indoline-2,3-dione, undergoes ready condensation with a variety of compounds with activated methylene groups. Derivatives of such compounds would provide useful substrates for photocyclizations which might afford diverse compounds of potential biological or medical interest. As a model compound for the study of some of these possibilities, 3-(3'-chloro-2'-oxopropyl)-3-hydroxyindoline-2-one (5) was synthesized and characterized. An improved synthesis of 5 was devised. Photolysis of 5 did not result in a simple photocyclodehydrohalogenation. A crystalline compound (6) was isolated in 20% yield and shown to be 3-acetyl-2,4-dihydroxyquinoline. Compound 6 had been formed by loss of HCl and a rearrangement. A reasonable working mechanism has been proposed for this rearrangement; other products have not been isolated as yet.

Several years ago in the course of some experiments carried out to determine the feasibility of the intermolecular photodehydrohalogenation reaction, anomalous phototitration results were obtained when phenyl chloroacetate was employed as one of the reactants. A literature survey revealed that phenyl chloroacetate apparently undergoes a photo-Fries rearrangement to form ortho-hydroxyphenacyl chloride, which undergoes photo-induced dehydrohalogenation to yield coumaran-3-one in 40% yield [Anderson and Reese, Tetrahedron Letters, 1 (1962)]. Such a photo-induced rearrangement and photocyclodehydrohalogenation may afford a useful, mild and specific

method for synthesizing compounds of biological interest or for modifying compounds of biological or medical interest possessing the aromatic hydroxyl moiety. As a beginning, to investigate these possibilities and to determine the course of the reaction in the naphthalene series, α- and β-naphthyl chloroacetates have been prepared. The preliminary results of a series of small-scale photolyses under various conditions as determined by thin layer chromatography and uv spectroscopy are promising. On the basis of these results reactions will be carried out on the preparative scale.

Significance to Biomedical Research and the Program of the Institute: New compounds produced in this work are of biological and medical interest. The organic chemical and photochemical reactions being developed can be applied to the synthesis of other compounds of biological and medical interest, to the modification of compounds of biological and medical interest, and to the development of methods for use in the study of biological and medical problems.

Proposed Course of Project: To continue to study the mechanisms of the reactions described in this report and to determine the extent to which they can be applied to related compounds, to related types of compounds and particularly to compounds of biological interest. To apply the methods developed to the synthesis of compounds of particular biological or medical interest. To develop and study other photochemical and organic chemical reactions and techniques with potential utility for the synthesis, modification, characterization, and study of molecules of biological and medical interest. To develop chemical methods which can be applied in the study of biological and medical problems.

Honors and Awards: none

Publications:

Foltz, C. M. and Kondo, Y.: A Novel Rearrangment Produced by the Photolysis of 3-(3'-Chloro-2'-oxopropyl)-3-hydroxyoxindole. Tetrahedron Letters 36: 3163-3166, 1970.

Foltz, C. M.: Organic Photochemistry. I. The Synthesis of 2-Oxo-2,3,4,5-tetrahydro-1H-naph[1,8-de]azocine by the Photolysis of N-Chloroacetyl-2-(α-naphthyl)ethylamine. J. Org. Chem. 36: 24-27, 1971.

Serial No. NIAMD-LC-46
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Structure Determination of N,N,N'-Trimethylsolanocapsine by X-ray Crystallography

Previous Serial No.: None

Principal Investigator: John A. Beisler

Other Investigators: Y. Sato (NIAMD-LC) and J. V. Silverton (NHLI-LC)

Cooperating Unit: None

Man Years

Total: 0.45
Professional: 0.2
Other: 0.25

Project Description:

Objective: To determine the crystal and molecular structure of a trimethyl derivative of the Solanum pseudocapsicum alkaloid, solanocapsine, by x-ray crystallography.

Methods Employed and Major Findings: The N,N,N'-trimethyl derivative of solanocapsine gives well-formed, monoclinic prisms from DMF-MeOH solution. Precession photographs established the space group as $P2_1$ by systematic absences and the cell constants as: a = 14.69(2), b = 7.39(2), c = 14.69(3) Å and $\beta = 115°20'$.

Significance to Biomedical Research and the Program of the Institute: Solanocapsine suppresses the growth of mycobacterium leprae and M. tuberculosis. With both nitrogen atoms quaternized the alkaloid has activity as a neuromuscular blocking agent.

Proposed Course of Project: Plans are in progress to collect x-ray diffraction data from a crystal of N,N,N'-trimethylsolanocapsine and initiate the phase problem solution.

Honors and Awards: None

Publications: None

Serial No. NIAMD-LC-47
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Structure Determination of Cedrone by X-ray Crystallography

Previous Serial No.: NIAMD-LC-47

Principal Investigator: John A. Beisler

Other Investigators: H. M. Fales and J. V. Silverton, NHLI-LC

Cooperating Unit: None

Man Years

Total: 0.2
Professional: 0.2
Other: 0

Project Description:

Objective: To determine the structure of cedrone through the utilization of x-ray crystallography.

Methods Employed and Major Findings: Trimethylphloroglucinol reacts with mild oxidizing agents to give a dimer which could not be unambiguously assigned a structure on the basis of chemical and spectroscopic analysis.

Cedrone crystallizes from DMF as solvated monoclinic prisms in space group $P2_1/c$. A phase solution was obtained using the symbolic addition method of Hauptman and Karle. Refinement of the resulting structure proceeded smoothly to a final R-factor of 0.058. Cedrone DMF solvate was revealed as a symmetrical, highly-strained pentacyclic structure wherein two molecules of DMF are directed toward it under the influence of two strong hydrogen bonds. Three carbon-carbon bonds proved to be exceptionally long: 1.597, 1.597, and 1.577 Å.

Significance to Biomedical Research and the Program of the Institute: The *in vitro* dimerization of trimethylphloroglucinol is analogous to oxidative phenolic coupling often found as a result of plant metabolism.

Proposed Course of Project: Since the objectives of this project have been met, no further work is planned.

Honors and Awards: None

Publications:

Beisler, J. A., Silverton, J. V., Penttila, A., Horn, D. S., and Fales, H. M.: The structure of "symmetrical cedrone." J. Am. Chem. Soc. (in press).

Beisler, J. A. and Silverton, J. V.: The crystal and molecular structure of symmetrical cedrone, a dimeric oxidation product of trimethylphloroglucinol. Acta Cryst. (in press).

Serial No. NIAMD-LC-48
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Degregation and Structural Elucidation of the Triterpenoid, Carpesterol.

Previous Serial No.: NIAMD-LC-45

Principal Investigator: John A. Beisler

Other Investigator: Yoshio Sato

Cooperating Unit: J. V. Silverton, Georgetown University

Man Years

Total: 0.2
Professional: 0.2
Other: 0

Project Description:

Objective: The structure determination by x-ray diffraction of carpesterol, a triterpenoid from Solanum xanthocarpum, and an investigation of the chemistry and chemical degradation of the natural product.

Methods Employed and Major Findings: The p-iodobenzesulfonate derivative of carpesterol gave triclinic plates, space group, P1, suitable for collection of x-ray diffraction intensity data. Although an electron density map phased with sulfur and iodine contributions showed a pseudocenter of symmetry, an adequate phasing model was obtained via seven cycles of weighted structure factor calculations followed by electron density maps. Subsequent least-squares refinement of the structure gave an R-factor of 8.9%. The structure of carpesterol was found to be (22R)-22-hydroxy-6-oxo-3β-benzoyloxy-4α-methyl-5α-stigmast-7-ene.

Methods have been found to degrade carpesterol to the known 4α-methyl-stigmast-8(14)-en-3β-ol. This identity was used to confirm the (24R)-configuration of the stigmasterol ethyl group.

Significance to Biomedical Research and the Program of the Institute: Carpesterol, as an elaboration of a (24R)-24-ethyllophenol skeleton, logically supports suggested phytosterol biogenetic schemes. The disclosure of a 22-OH function in carpesterol suggests a biogenetic relationship with solasodine, which is spiroaminoketalized at C-22, and which is the principal

steroidal alkaloid of S. xanthocarpum.

Proposed Course of Project: After submitting the chemical aspects of carpesterol for publication, this project will stand as complete.

Honors and Awards: None

Publications:

Tsay, Y-H., Silverton, J. V., Beisler, J. A., and Sato, Y.: The structure of carpesterol. J. Am. Chem. Soc. 92: 7005-7006, 1970.

Serial No. NIAMD-LC-49
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The von Braun Degradation of Demissidine

Previous Serial No.: NIAMD-LC-46

Principal Investigator: John A. Beisler

Other Investigator: Yoshio Sato

Cooperating Unit: None

Man Years

Total: 0.2
Professional: 0.2
Other: 0

Project Description:

Objective: Solanidine Acetate and demissidine acetate can be degraded with cyanogen bromide in good yield to give bromocyanamides.

Methods Employed and Major Findings: The chemistry of the solanidanol bromocyanamides is characterized by the ease with which the parent alkaloid is regenerated under neutral, or even basic, hydrolysis conditions. The retro-von Braun reaction is interpreted as indicating an exceptional stability of the indolizidine ring system which is incorporated into the solanidane class of steroidal alkaloids.

Significance to Biochemical Research and the Program of the Institute: The aza-steroids formed by the von Braun reaction could be used as intermediates for the formation of products for antitumor and antihypertensive evaluation.

Proposed Course of Project: This project is now complete.

Honors and Awards: None

Publications:

Beisler, J. A., and Sato, Y.: Chemistry of the solanidane ring system. J. Chem. Soc., Section C (in press).

the synthetic procedures and screen results of the above mentioned analogs, this project is to be terminated.

Honors and Awards: None

Publications:

Beisler, J. A.: A short synthesis of several gambir alkaloids. Tetrahedron 26: 1961-1965, 1970.

Fry, E. M., and Beisler, J. A.: The sodium borohydride reduction of indolylethylpyridinium bromides, Hexahydroindoloquinolizines. J. Org. Chem. 35: 2809-2811, 1970.

Beisler, J. A.: Notiz über eine einfache synthese des alstonilins. Chem. Ber. 103: 3360-3361, 1970.

Serial No. NIAMD-LC-50
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Synthesis of Camptothecin Analogs

Previous Serial No.: NIAMD-LC-44

Principal Investigator: John A. Beisler

Other Investigator: E. M. Fry

Cooperating Unit: CCNSC

Man Years

Total: 0.2
Professional: 0.2
Other: 0

Project Description:

Objective: The alkaloid, camptothecin, is being clinically evaluated as an antitumor agent. Because of encouraging preliminary clinical results and because of a limited supply from natural sources, a synthesis program suggested itself.

Methods Employed and Major Findings: A synthetic scheme based on the postulated biosynthesis of camptothecin and a new synthesis of tetrahydro-β-carbolines has thus far proven to be effective in achieving the objectives of this project. Accordingly, the utility of the latter-mentioned new synthesis has been demonstrated in the synthesis of several gambir alkaloids as well as the alkaloid, alstoniline. Relevant carbazole derivatives have been successfully converted to indeno[1,2-b]quinoline structures by chemical means in imitation of the postulated biosynthetic pathway.

Thus, this preliminary work has succeeded in providing a foundation on which it should be possible to assemble structures very similar to camptothecin, and perhaps, the alkaloid itself.

Significance to Biochemical Research and the Program of the Institute: Some of the above mentioned synthetic alkaloids and a number of synthetic intermediates and analogs have been submitted to CCNSC for evaluation against a lymphoid leukemia screen.

Proposed Course of the Project: After preparation for publication of

LABORATORY OF EXPERIMENTAL PATHOLOGY

Introduction

The research activities in our Laboratory are summarized below and, as in the last year, progress in research areas are presented rather than individual activities, whenever this is possible.

The Laboratory has continued to provide tissue diagnoses on surgically obtained specimens and on selected autopsy material for the Division of Indian Health of the Public Health Service, the Hospital of the Coast Guard Academy in New London, Connecticut and to several of the Federal Prisons. In this activity, we were most fortunate to receive the generous help of several colleagues within NIH who are pathologists. We have thus continued to provide this service without seriously interrupting individual research. Those who helped us again, as in the past, were: Dr. Ruth Kirschstein (Chief of Pathology, DBS), Dr. Allen Cheever (Chief of the Section on Host-Parasite Relations, LPD-NIAID) and Dr. Albert H. Gelderman (LPD-NIAID).

Dr. Benjamin Highman has continued as the Liaison Officer of the Public Health Service to the Armed Forces Institute of Pathology. In spite of this added activity, Dr. Highman is actively conducting collaborative research here at NIH.

Biology of Degenerative Joint Diseases (Dr. Sokoloff and staff)

The Section on Rheumatic Diseases has extended its long-term study of the biology of degenerative joint disease. This condition centers about the interplay between aging and mechanical use in the wear and tear of joints.

Dr. Simon has concluded a series of biomechanical studies of joints. He investigated for the first time the wear properties of articular cartilage *in vitro* using a reversed steel abrader ball and found that the principal determinants were surface friction as well as intrinsic properties of the tissue (strength and stiffness). Synovial fluid had a distinct wear-protecting effect; this was eliminated by digestion of the mucin with trypsin but not hyaluronidase, thus extending previous but surprising experience about viscosity having no effect on lubrication of joints. In another paper, it was reported that thickness and intrinsic elasticity make independent contributions to the deformability of articular cartilage, and this is interpreted in terms of increasing congruence of joints surfaces, so diminishing stresses on the joint surface.

With Dr. Green, he found that the material properties of joint cartilage, as gauged by resistance to withstand abrasion over the course of six months *in vivo* was not compromised by death of the cells. The latter was induced by cryoprobe injury which selectively killed the chondrocytes without altering the matrix. This places in better perspective a large literature in which other mechanical means or cautery was used to induce osteoarthritic lesions, and necrobiosis of the cells indicted.

A pituitary growth factor for articular chondrocytes was discovered by Drs. Corvol and Sokoloff along with Mr. Malemud. The active principle is associated with the glycoprotein hormones, thyrotropic and luteinizing hormones, but is not itself TSH as shown by more highly purified preparations. It is not growth hormone, sulfation factor or exophthalmos-producing substance. A similar mitogenic effect was found with human chorionic gonadotropin, which has many similarities to the preceding. The association of these compounds with the response observed suggests that this cartilage factor may also be a glycoprotein and conceivably a hormone.

Amyloidosis (Dr. Glenner and staff)

The studies on amyloidosis have made great progress during the past year and are summarized here in somewhat greater detail.

Human amyloidosis: Amyloidosis is a pathologic condition in which a fibrillar protein, amyloid, is deposited extracellularly in various organs of the human body leading to metabolic disturbances in the involved tissues (e.g. heart, liver, kidney). The identifying characteristics of native human amyloid fibrils are their green polarization birefringence after staining with Congo red, constant tryptophan content, fibrillar appearance when examined by electron microscopy and x-ray diffraction crystallographic pattern revealing a "β-pleated sheet" configuration (Eanes and Glenner). In order to identify and characterize the major protein constituents of amyloid fibrils, a method for fractionation of denatured amyloid fibril concentrates by gel filtration in guanidine-HCl was recently described (Glenner, et al). The major protein of amyloid fibril concentrates of ten tissues obtained from six patients was fractionated by sequential gel filtration on Sepharose 4 B and Sephadex G-100 columns using 5 M guanidine-HCl in 1 N acetic acid with removal of over 28% of minor constituents. The major amyloid protein from each individual differed from that of the others in molecular weight, amino acid composition and peptide map profile. The major protein from the liver and spleen of the same individual, however, were identical. A serum component having an immunologic relationship to the patients' major amyloid protein was detected migrating to the γ-globulin region on immunoelectrophoresis. In view of the marked chemical similarity of amyloid protein and immunoglobulin protein, e.g. unreactive or Asx N-terminal groups, constant tryptophan content, heterogeneity between individuals and homogeneity within the individual, and the molecular weight ranges of amyloid fibril protein found, the possibility that amyloid might be derived from the N-terminal variable segment of the light or heavy chain of immunoglobulin proteins (e.g. the Fd fragment) was considered.

In order to test this postulate directly and conclusively, the amino-terminal amino acid sequencing of one of the purified amyloid proteins was undertaken using an automatic amino acid sequencer, Beckman Model 890, following the method of Edman and Begg. The sequence of this amyloid protein was found to be

Asp-Ile-Gln-Met-Thr-Gln-Ser-

This amino-terminal amino acid sequence categorized this amyloid protein as belonging to a subgroup of immunoglobulin light polypeptide chains similar to kappa I. Further sequencing of this protein to position 36 and of another amyloid protein to position 35 revealed both to be homologous to a previously sequenced Bence-Jones κ-protein of variable region subgroup $V_{\kappa I}$. These amyloid proteins are, therefore, portions of κ-type light chains of the $V_{\kappa I}$ variable region subgroup. The molecular weight of an intact light polypeptide chain is approximately 22,500. The molecular weights of 7,500 and 18,300 for these amyloid proteins, as determined by both SDS polyacrylamide gel electrophoresis and calibrated Sephadex G-100 columns, indicate that these proteins are not complete light chains. Since both proteins start with the appropriate amino-terminal sequence, it is possible that these represent light chain fragments with varying amounts of the constant portion degraded. Alternatively, amyloid may consist of light polypeptide chains having internal deletions of varying lengths affecting variable regions, constant regions, or both, such as those reported in "heavy chain disease" proteins.

The losses on purification and the yield on sequence analysis (when compared with known homogeneous light chains) indicate that the proteins sequenced are the major components of amyloid and are not minor contaminants. Since only 30% of the protein content of the fractionated amyloid fibril concentrate is lost in purfication and since sequence analysis and disc gel electrophoresis give evidence of the presence of a single monomeric component in the final preparation, the possibility that non-specifically absorbed homogeneous immunoglobulins accounted for 70% of the amyloid fibril concentrate would appear highly unlikely. The major protein component of these amyloid preparations is, therefore, a portion of an immunoglobulin light polypeptide chain and amyloidosis is, therefore, a disease caused by the deposition in tissues of an amino-terminal fragment of the variable region of the light chain of homogeneous immunoglobulins by a mechanism presently under investigation.

The above findings not only describe the nature of amyloid, but provide a method of isolation of a homogeneous portion or fragment of immunoglobulins believed to be important in antigen-binding, one of the first steps in the bodily defense mechanism for removing bacteria and other foreign proteins. This leads to the possibility of a better understanding of the mechanism of this crucial step in the body's defense against disease. In addition these findings indicate that amyloidosis is the final common pathway of what may be a variety of synthetic or degradative disorders of immunoglobulin metabolism. It is one of the few diseases of immunoglobulin metabolism in which the nature of the lethal component has been defined on a molecular basis (Drs. Glenner, Page, Isersky, Harada, Bladen, Terry and Eanes).

Experimental amyloidosis: Murine amyloid has been produced by four different induction methods (Mycobacterium butyricum, casein, casein plus Freund's adjuvant and endotoxin-induced mouse amyloidosis) in several strains and obtained from mice with "spontaneous" amyloidosis. The amyloid fibrils have been concentrated from spleen and liver. Electron microscopy of all of these

preparations reveals the amyloid fibril to be 100 Å in width and composed of two parallel filaments, each measuring 35-40 Å in width and having the appearance of a twisted ribbon. X-ray diffraction of all preparations reveals a "backbone" spacing at 4.75 Å and a "side chain" spacing at 11 Å indicating a "β-pleated sheet" structure. Identification of the major protein component of amyloid fibril concentrates was made by combined use of sodium dodecyl sulfate polyacrylamide disc electrophoresis, labeling of the protein with ^{3}H-tryptophan and Sepharose 4 B gel filtration. Purification of the amyloid protein from spontaneous amyloidosis liver was accomplished by sequential gel filtration with 5 M guanidine in 1 N acetic acid on Sepharose 4 B and Sephadex G-100 or G-75 columns. The material is a unique protein with a molecular weight of 7200, a high content of dicarboxylic and short chain amino acids, a significant amount of tryptophan and an unreactive amino-terminal amino acid. Murine amyloid protein, therefore, has striking similarities to many human amyloid protein preparations. It differs from the human proteins in the similarity of molecular weights of different preparations. Since human amyloid protein has been found to be derived from the amino-terminal variable fragment of the light polypeptide chain of immunoglobulins, an investigation of murine amyloid fibril protein, to determine if it has a similar origin, has been undertaken. Preliminary evidence indicates that one of the murine amyloid proteins is derived from a λ-light chain. Evidence from this study indicates that the experimental animal model is a valid counterpart of the human disease and will be of value in defining the pathogenesis of amyloidosis.

A detailed study of "spontaneous" amyloidosis in mice has revealed that this process, previously attributed to aging factors, is the direct result of chronic inflammation consequent to aggressive behavior of male mice in a colony. This study emphasizes the necessity of investigating all cases of so-called idiopathic or "primary" amyloidosis in humans in order to determine if an underlying infectious process or metabolic derangement is a predisposing factor for the development of amyloidosis in any case of this disease (Drs. Glenner, Page, Isersky, Harada, Bladen, Terry and Eanes).

Cytogenetic Studies (Dr. Tjio and staff)

Cytogenetic studies on the effect of LSD: A continuing double-blind, controlled research project is in progress on the effects of LSD on human peripheral blood chromosomes. Current series of patients, who are receiving LSD as part of their psychiatric treatment at Spring Grove State Hospital in Baltimore, has been expanded to include 25 persons given DPT (another hallucinogen). As before, two pretreatment and two posttreatment blood samples are drawn on different days from each patient. Although the coded samples cannot be identified until the study is complete, no cultures have been found with a chromosome aberration rate greatly in excess of rates considered normal for the general population. This trend again supports previous work (with Dr. Pahnke, Maryland Psychiatric Research Center).

Studies using the mixed leukocyte reaction to examine cellular immunity in vitro: Blastogenesis occurs when peripheral blood leukocytes of unrelated individuals are cultured together. This "mixed leukocyte reaction" is a function of the degree of genetic compatibility between two people's cells.

It has been determined that there are pairs of siblings whose cells give no detectable reaction together but who are makedly different in their response to some antigen. Experiments are planned in which cultures containing various combinations of lymphocytes, purified in nylon-fiber columns, and glass-adherent cells (mostly macrophages) from two such donors are incubated in the presence of the appropriate antigen. The origin of the responding cells can be determined by chromosome analysis if the donors are of different sexes; this should aid in the identification of the roles of the cells involved in the immune response.

Cytogenetic studies on T1Wh translocation mouse strain:

A. Evaluation of fertility: Reduction of litter size of T1Wh homozygotes with inbreeding has continued. When the strain was first developed, litter size at birth was 7.1. Average size of 35 litters of F 13 and 14 during the past year was 5.9. The inbreeding program is now in generation 15.

B. Meiotic and other studies of mouse hybrids: Meiotic studies of hybrids of T1Wh and other strains with Robertsonian fusion translocations (T1A1d, T163) are in progress. These indicate that the translocations T1Wh and T163 have a single small chromosome in common, but that the chromosomes involved in the T1A1d translocation are not common to T1Wh or T163. Attempts are now being made to further reduce the mouse chromosome number from 38 to 36 by breeding together hybrids carrying both T1Wh and T1A1d chromosomes. If animals homozygous for these 2 translocations are obtained by means of these F_1 crosses or backcrosses to the parental strains, they will be used to initiate a new mouse strain carrying 36 chromosomes. Chromosome studies of mouse embryos and fetuses from crosses of the various strains are also in progress. These are part of an overall study of the segregation of hybrid translocation chromosomes and its relationship to frequency of aneuploidy among hybrid offspring.

C. Autoradiographic studies: Preparations of spleen cells from T1Wh homozygotes and normal mice did not reveal any distinctive labeling pattern of the translocation chromosomes or other mouse autosomes. Labeling of normal and translocation animals was diffuse and nonspecific. Labeling studies of the other translocation strains may be attempted in the future.

D. Transfer of cells across the mouse placenta: Chromosome preparations of fetal and maternal tissues at various stages of gestation and after delivery, utilizing the T1Wh chromosome as a marker to differentiate maternal from fetal mitoses, are complete. Chromosome preparations of various tissues of 44 offspring of 7 pregnancies did not reveal presence of maternal mitoses after screening of 14,396 cells. Screening of 5,902 maternal mitoses from lung, marrow, and spleen of 9 pregnant and postpartum mice revealed no fetal cells. These negative findings are consistent with the majority of studies now reported in the literature.

Relationship of viral DNA to mouse chromosomes: Preparations of MVM (minute virus of mice) labeled with H^3-thymidine have been added to cultures of normal mouse spleen cells in order to study the possible association of the viral DNA

with host chromosomes. Blind analysis of autoradiographic preparations of these cultures and their controls exposed for 3 and 24 hours before harvest was done. The intensity of labeling (number of grains per metaphase localized over chromosomes) was similar in virus and control groups, and background was low in both cases. Position of grains (end or middle of chromatid, or at centromere) was observed in both groups, while there were differences in grain distribution between control and MVM preparations, further studies using a higher multiplicity of virus will be necessary to evaluate these. The previous study utilized a ratio of 5 viruses per cell (Drs. Tjio and White).

Structure and Biochemical Properties of Cell Membranes (Dr. Marchesi and staff)

Further characterization of the principal glycoprotein of the human red cell membrane: A single glycoprotein can be isolated from red blood cells by treating ghost membranes with lithium diiodosalicylate (Marchesi, Fed. Proc. 29: 600, 1970). The purified molecule appears homogeneous, has A, B, and MN blood group activities and the carbohydrate receptors for influenza virus, phytohemagglutinin, and wheat germ agglutinin. Trypsin hydrolysis of the protein yields two classes of glycopeptides (α and β) which have been partially purified by a combination of gel filtration and chromatography on Dowex 50-X2 and DEAE-cellulose. Both glycopeptides have a molecular weight of approximately 15,000 but differ in amino acid compositions and their mobilities on acrylamide gel electrophoresis. Both have the receptors for influenza virus and phytohemagglutinin. Cyanogen bromide cleavage of the glycoprotein yields glycopeptides and peptides containing no detectable carbohydrate. One of the latter peptides has a molecular weight of approximately 12,000 and contains the bulk of the hydrophobic amino acids of the original glycoprotein. Trypsin digestion of intact red blood cells results in the preferential release of the α glycopeptides. The β glycopeptides are released by trypsin only after the modified glycoprotein (minus α) has been isolated. These results suggest that the glycoprotein is a single polypeptide chain composed of two glycopeptide portions and a hydrophobic segment (with R. L. Jackson and J. Segrest).

Role of glycoproteins in membrane structure: It has been suggested that glycoproteins on the red cell surface are oriented with their carbohydrate-rich portion exposed to the exterior and hydrophobic segments embedded in the lipid of the membrane (Morawiecki, Winzler). This view is based primarily on the results of proteolytic digestion of intact red cells and on trypsin treatment of isolated glycoproteins. Direct evidence in support of this model has been obtained which relates the glycoprotein of the red cell membrane to 75 Å globular particles which are located within the lipid portion of the membrane.

The distribution of glycoproteins on the surfaces of intact red cells and ghosts was mapped by studying replicas of freeze-etched preparations by electron microscopy. Although the exposed portions of glycoprotein molecules are too small to be seen by this technique, specific sites on the molecules can be labeled by markers (Influenza virus, phytohemagglutinin, or ferritin-conjugated phytohemagglutinin) which are easily recognized. The distribution of these markers indicates that the glycoproteins are dispersed uniformly over

the surfaces of the cells in the same distribution as the intramembranous particles. Trypsin treatment of red cell ghosts results in a rearrangement of the intramembranous particles, and the labeled sites on the outer surface also shift to correspond to the new particle pattern.

It is not yet clear how the glycoproteins are connected to the intramembranous particles. One idea is that hydrophobic parts of the molecules interact with lipids and/or proteins, and together this complex forms the globular unit (with T. W. Tillack and R. E. Scott).

A new method for the isolation of tumor-specific antigens from human neoplasms: Carcinomas of the gastrointestinal tract have a membrane-bound glycoprotein (called carcinoembryonic or CEA antigen) which is not detectable in normal adult tissue. This tumor-specific protein can also be assayed immunologically in the sera of patients with gastrointestinal carcinomas (Thomson et al., Proc. Nat. Acad. Sci. 64: 161, 1969), thus providing a new method for the early diagnosis of cancer. The original method for the isolation of CEA antigen involves extraction of tumors with perchloric acid and fractionation of acid-soluble glycoproteins by electrophoresis and gel filtration. We have developed a new method using lithium diiodosalicylate (LIS), which has a number of advantages: LIS dissociates membranes into their macromolecular components under relatively mild conditions, and the entire glycoprotein components of the membranes can be extracted in water-soluble form without the use of perchloric acid.

A glycoprotein, extracted with LIS from human colonic tumors, has been purified by cold phenol extraction, ion exchange chromatography, gel filtration and isoelectric focusing. It is homogeneous by acrylamide gel electrophoresis and is immunologically identical with the original CEA antigen supplied by P. Gold. This technique is effective in extracting membrane glycoproteins from other cell types and is now being used to isolate membrane proteins from other human tumors (with J. Rosai and T. W. Tillack).

Histochemical and Ultrastructural Studies: Methods and Applications (Dr. Feder and staff)

Microperoxidase as an ultrastructural tracer: Ultrastructural studies have been carried out with microperoxidase (MP), a heme-peptide developed as a tracer in this laboratory. After introduction of this compound into tissues or cells, its position can be readily determined with the electron microscope. The unusual feature of MP is its small size; of the ultrastructural tracers that can be used in vivo, MP has the lowest molecular weight (about 1900). Several studies have been carried out with MP, as follows: (a) The electrotonic synapse in the giant axon of the crayfish is an easily studied example of the gap junction, a common type of cell-to-cell contact. This synapse is permeable to MP injected intracellularly, but not to horseradish peroxidase (mol. wt. 40,000). The substructure of the synapse and the route of transmission from cell to cell via the gap junction are being studied by means of MP (Drs. Feder, Reese, and M. V. L. Bennett). (b) Continuity between the periaxonal space and the interstitial space of the brain has been demonstrated

with MP (Drs. Feder, Reese, and Brightman). (c) A major anatomical correlate of the physiological blood-brain barrier is a continuous band of tight junctions in the capillary endothelium of the brain. This conclusion, previously reached as a result of observations made with horseradish peroxidase, has now been confirmed with MP (Drs. Feder, Reese, and Brightman). (d) The "blood-testis barrier," as delineated by horseradish peroxidase and MP, appears to be located near the base of the seminiferous epithelium in a continuous band of tight junctions formed by Sertoli cells with each other (Drs. Feder and W. I. Bennett).

Thyroid peroxidase cytochemistry: The apparent localization of thyroid peroxidase was found to be affected by the duration of fixation, by the rate of endogenous hydrogen peroxide production, and probably also by the presence of diaminobenzidine, which is usually a component of the cytochemical peroxidase medium (Drs. Tice and Wollman).

Fine structure of tissues and cells: thyroid and eosinophil: (a) Chronic hypercalcemia induced by parathyroidectomy has been found to cause an increase in the number of light cells, in the cell volume, and in the amount of material in the secretory vesicles of the light cell. These findings provide further evidence that the light cell contains thyrocalcitonin (Drs. Wetzel and Gittes). (b) Comparative fine structural studies of eosinophil granulogenesis in mammals have borne out earlier suggestions (Wetzel, 1970b) that most species display a pattern resembling that of rabbits, rats, mice, guinea pigs, and humans. However, the following exceptions have been elucidated: 1) In cats all eosinophil granules, even in the earliest cells, contain dense lamellae that evidently aggregate to form the multilaminate whorls which characterize all mature eosinophil granules in this species. 2) In dogs most of the eosinophil granules are spherical and homogeneous, even in mature cells, and only a few extraordinary granules display dense crystalloids such as typify mature eosinophil granules of other mammalian species. These exceptional cases will prove helpful in proposed autoradiographic studies of eosinophil granule formation (Dr. Wetzel).

Studies on wildfire toxin: Wildfire toxin is produced by Pseudomonas tabaci and is higly toxic to plants. The cytotoxicity is presumed to be due to the inhibitory effect of the toxin on glutamine synthetase. The structure of the toxin, which was previously unknown, has now been elucidated. Wildfire toxin is a dipeptide yielding on hydrolysis the amino acids threonine and tabtoxinine; the tabtoxinine is present in the toxin as a β-lactam. The biological activity of the toxin may be due to the β-lactam ring--a group previously found in nature only in the penicillins, in cephalosporin C, and in the pachystermines (Dr. Feder and Mr. Stewart).

Bacterial Endocarditis After X-irradiation (Dr. Highman)

In an earlier study, it was found that a moderately high incidence of bacterial endocarditis could be induced in rats by giving them 2 whole-body exposures to 400 R at 4-day intervals, followed 3 and 4 days later by 2 intravenous injections of a broth culture of Streptococcus mitis. Administration

of 4 mg/kg epinephrine in oil, an alpha and beta adrenergic agent, a day before each of the 2 successive daily bacterial inoculations increased greatly the incidence and severity of the endocarditis in the irradiated animals. It was postulated that this synergistic effect was due to severe impairment of the cellular and humoral defense mechanisms caused by a combination of severe leukopenia, induced by the x-irradiation, and local circulatory stasis and hypoxia resulting from a severe myocarditis and prolonged vasoconstriction induced by the epinephrine. To test this hypothesis further, 40 mg/kg of isoproterenol, a beta adrenergic agent, was given in place of epinephrine in oil immediately before each bacterial inoculation. Isoproterenol increased the incidence and severity of endocarditis to a degree comparable to that produced by epinephrine, suggesting that the effect of epinephrine may have been due to its beta adrenergic action.

Exercise Tolerance in Cold and Altitude Acclimated Rats (Dr. Highman)

In an earlier study, rats acclimated to cold (1.7°C) and unacclimated rats were exercised at room temperature by being required to walk for several hours in a rotating cylindrical cage. The acclimated rats developed after exercise higher serum elevations of certain enzymes such as glutamic oxalacetic transaminase and tolerated exercise less (became fatigued earlier) than unacclimated rats. In a more recent study, rats were similarly exercised 3-9 hours in a cold room maintained at 1.7°C, and two-thirds of a group of unacclimated rats died during such a 9-hour exposure. All cold acclimated rats survived; and they tolerated exercise better and showed a smaller rise in serum enzyme levels after exercise than unacclimated rats. These findings indicate that a dual stress has a deleterious effect, that room temperature is a dual stress to the exercising cold acclimated rat, that cold is a dual stress to the exercising unacclimated rat, and that prior acclimation to one of the dual stresses is helpful. These studies are important because human exposure to such multiple environmental stresses is common.

In another study, no significant cardiac lesions and no reduction in exercise tolerance were noted in rats with polycythemia induced by feeding a diet containing cobaltous chloride or by repeated daily exposures to 18,000 ft simulated high altitude. In rats exposed 5-hours daily to 25,000 ft for 2-6 weeks, however, exercise tolerance was greatly reduced and foci of myocardial necrosis and fibrosis were often found in the left periventricular apical myocardium. Repeated bleeding during 6 weeks at 25,000 ft prevented polycythemia and a reduction in exercise tolerance and minimized pathologic changes. After discontinuance of the exposures to 25,000 ft the apical lesions persisted, but exercise tolerance gradually returned to normal. These findings indicate that severe hypoxia induced by exposure to 25,000 ft is the principal factor responsible for myocardial damage and reduced exercise tolerance and that severe polycythemia is a dual stress and requisite secondary factor (Drs. Highman, Altland, Dieter, and Maling).

Cycasin Research (Dr. Laqueur)

The studies outlined in last year's summary have continued. Additional animals have been prepared for observations of long-term effects of prenatal

retinal injury. Thus far no changes have been seen to suggest malignant transformation in the retina. A comparative study on the effects of single subcutaneous and intraperitoneal injections of MAM acetate in newborn and young germfree rats on tumor incidence has been completed for groups of animals treated early in life (5, 9, and 16 days old at time of injection). The study has been extended to 25 and 35 day old animals because a 100 percent tumor incidence was found up to and including the 16th day. This study is being undertaken to test in vivo the validity of previous findings that the enzyme responsible for hydrolysis of the β glucoside cycasin indeed disappears from the tissues as the animal matures. A study has been initiated to examine by histochemical techniques the distribution of β-glucosidase in tissue during late fetal and early postnatal life with the expectation that we might learn something about the physiological significance of this enzyme which at present is unknown.

Serial No. NIAMD-LEP-1
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Metabolic and carcinogenic effects of Cycas circinalis, its glucoside cycasin and its aglycone, methylazoxymethanol, in conventional and germfree rats.

Previous Serial Number: Same

Principal Investigator: Gert L. Laqueur

Other Investigators: Dr. M. Spatz, Dr. H. Matsumoto, and Mr. E. G. McDaniel

Cooperating Units: LNNS-NINDS (Dr. Spatz)
LNE-NIAMD (Mr. McDaniel)
University of Hawaii (Dr. Matsumoto)

Man Years:

Total: 4
Professional: 1
Others: 3

Project Description:

Studies outlined in last year's report have continued. The experiments dealing with prenatally induced retinal injury have been enlarged to study the late effects of this retinopathy on visual acuity and possible tumor induction. Although the acute and subacute changes are by now well known, the late effects require extended studies.

A second study under way is concerned with an in vivo investigation of the transient nature of β-glucosidase activity in the tissues of rats during the perinatal period. Past biochemical investigations on selected tissues (subcutaneous tissue and small intestine) suggest that this enzyme disappears on about the 20th postnatal day. To test for this enzymatic activity germfree rats were injected at various periods after birth with the β-glucoside cycasin. The incidence of tumors obtained in the different groups should indicate how long after birth cycasin can be hydrolyzed to the carcinogen methylazoxymethanol in the living organism. The results thus far are complete for those groups of rats injected at 5, 9 and 16 days, all of which had tumors; those injected at 25 and 35 days after birth are still alive. In conjunction with these in vivo studies, histochemical investigations are now in preparation to examine

the localization and distribution of this enzyme in fetal and neonatal tissues in the hope to learn something about the physiologic significance of this enzyme which is presently unknown.

Publications:

Laqueur, G. L.: Contribution of intestinal macroflora and microflora to carcinogenesis. In: Burdette, W. J. (Ed.): Carcinoma of the Colon and Antecedent Epithelium. Springfield, Ill., Charles C. Thomas Co., 1970, pp. 305-313.

Laqueur, G. L.: Eosinophilic meningitis (Angiostrongylus cantonensis). In: Marcial-Rojas, R. A.(Ed.): Pathology of Protozoal and Helminthic Diseases with Clinical Correlation. Baltimore, Md., Williams and Wilkins Co., 1971, pp. 756-759.

Laqueur, G. L.: Pathogenicity of cycasin. Acta Path. Jap. (in press).

Serial No. NIAMD-LEP-2a
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Histopathologic, serum enzyme and other changes produced in animals by various environmental and other stresses.

Previous Serial Number: Same

Principal Investigator: Benjamin Highman

Other Investigators: Drs. P. D. Altland, M. P. Dieter, H. M. Maling

Cooperating Units: LPB-NIAMD (Altland, Dieter - LBP-20)
LCP-NHLI (Maling)

Man Years

Total: 1.5
Professional: .5
Other: 1

Project Description:

Exercise Tolerance in Cold and Altitude Acclimated Rats: In an earlier study, rats acclimated to cold (1.7°C) and unacclimated rats were exercised at room temperature by being required to walk for several hours in a rotating cylindrical cage. The acclimated rats developed after exercise higher serum elevations of certain enzymes such as glutamic oxalacetic transaminase and tolerated exercise less (became fatigued earlier) than unacclimated rats. In a more recent study, rats were similarly exercised 3-9 hours in a cold room maintained at 1.7°C, and two-thirds of a group of unacclimated rats died during such a 9-hour exposure. All cold acclimated rats survived, and they tolerated exercise better and showed a smaller rise in serum enzyme levels after exercise than unacclimated rats. These findings indicate that a dual stress has a deleterious effect, that room temperature is a dual stress to the exercising cold acclimated rat, that cold is a dual stress to the exercising unacclimated rat, and that prior acclimation to one of the dual stresses is helpful. These studies are important because human exposure to such multiple environmental stresses is common.

In another study, no significant cardiac lesions and no reduction in exercise tolerance were noted in rats with polycythemia induced by feeding a diet containing cobaltous chloride or by repeated daily exposures to 18,000 ft simulated high altitude. In rats exposed 5-hours

daily to 25,000 ft for 2-6 weeks, however, exercise tolerance was greatly reduced and foci of myocardial necrosis and fibrosis were often found in the left periventricular apical myocardium. Repeated bleeding during 6 weeks at 25,000 ft prevented polycythemia and a reduction in exercise tolerance and minimized pathologic changes. After discontinuance of the exposures to 25,000 ft, the apical lesions persisted, but exercise tolerance gradually returned to normal. These findings indicate that severe hypoxia induced by exposure to 25,000 ft is the principal factor responsible for myocardial damage and reduced exercise tolerance and that severe polycythemia is a dual stress and requisite secondary factor.

Hepatoxic Studies: It is well known that a fall in plasma triglycerides and triglyceride accumulation in the liver precedes the development of hepatic necrosis after administration of carbon tetrachloride. Other compounds, such as thioacetamide and bromobenzene, also produce liver necrosis but their effects on plasma triglycerides have not been studied and are currently under investigation. The purpose of this study is to explore the possible correlations between decreased plasma triglyceride levels, elevated liver triglyceride levels, and the development of hepatic necrosis. The effects of phenobarbital induction of drug-metabolizing enzymes and pretreatment with various blocking compounds will also be determined to see whether potentiation or inhibition of triglyceride accumulation in the liver is associated with potentiation or inhibition of necrosis. Further details concerning this project and preliminary results are detailed elsewhere by Dr. H. M. Maling (LCP-NHLI).

Publications:

Dieter, M. P., Altland, P. D. and Highman, B.: Tolerance of unacclimated and cold-acclimated rats to exercise in the cold: serum, red and white muscle enzymes, and histological changes. Canadian J. Physiol. Pharm. 48: 723-731, 1970.

Altland, P. D., Highman, B., Parker, M. G. and Dieter, M. P.: Serum enzyme, corticosterone and tissue changes in rats following a single oral dose of ethanol. Quart. J. Studies Alcohol 31: 281-287, 1970.

Altland, P. D. and Highman, B.: Effect of polycythemia and altitude hypoxia on rat heart and exercise tolerance. Am. J. Physiol. (in press)

Serial No. NIAMD-LEP-2b
1. Armed Forces Institute of Pathology
2. Radiopathology Division
3. Washington

PHS-NIH-AFIP
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: 1. Experimental bacterial endocarditis following x-irradiation.
2. Serum enzyme and pathologic changes after whole-body irradiation and effect of partial shielding and various adrenergic, hepatotoxic and blocking agents.
3. Characterization of the anatomic, histologic, pathologic and selected physiologic attributes of Mystromys.
4. Radiological-pathological correlations

Previous Serial Number: Same

Principal Investigator: Benjamin Highman

Other Investigators: Capt. E. G. Theros, Cpt. N. T. Byers, Cpt. W. H. Cyr, Cpt. R. P. Streett and Dr. M. R. Rodrigues

Cooperating Units: Radiation Pathology Branch, AFIP (Byers, Cyr, Streett)
Radiologic Pathology Branch, AFIP (Theros)
Ophthalmic Pathology Branch, AFIP (Rodrigues)

Man Years

Total: 1.5
Professional: .5
Other: 1

Project Descriptions:

Bacterial Endocarditis After X-irradiation: In an earlier study, it was found that a moderately high incidence of bacterial endocarditis could be induced in rats by giving them 2 whole-body exposures to 400 R at 4-day intervals, followed 3 and 4 days later by 2 intravenous injections of a broth culture of Streptococcus mitis. Administration of 4 mg/kg epinephrine in oil, an alpha and beta adrenergic agent, a day before each of the 2 successive daily bacterial inoculations increased greatly the incidence and severity of the endocarditis in the irradiated animals. It was postulated that this synergistic effect was due to severe impairment of the cellular and humoral defense mechanisms caused by a combination of severe leukopenia, induced by the x-irradiation, and local circulatory stasis and hypoxia resulting from a severe myocarditis and prolonged vasoconstriction induced by the epinephrine. To test this hypothesis further, 40 mg/kg of isoproteronol, a beta adrenergic agent, was given in place of epinephrine in oil

immediately before each bacterial inoculation. Isoproterenol increased the incidence and severity of endocarditis to a degree comparable to that produced by epinephrine, suggesting that the effect of epinephrine may have been due to its beta adrenergic action. It should be noted that 40 mg/kg isoproterenol, like epinephrine, causes severe myocardial damage. Further studies are in progress to determine the effect of smaller doses of isoproterenol and pretreatment with adrenergic blocking agents and the effect of alpha adrenergic agents and other compounds on the susceptibility of irradiated rats to endocarditis.

Enzyme Changes: It is well known that administration of isoproterenol and various hepatotoxic agents such as carbon tetrachloride and ethanol induces visceral fatty changes and a rise in SGOT and certain other serum enzyme levels. Preliminary studies indicate that prior whole-body x-irradiation may alter the magnitude of these pathologic and serum enzyme changes, and that this varies in part with the irradiation dose and interval preceding administration of the drugs.

Studies on Mystromys Albicaudatus (the African White-Tailed Rat): Since the introduction of this South African rodent into this country in 1962, a large colony has been maintained at the AFIP. Its advantages as a laboratory animal include a longevity of about 6 years and freedom from certain infections such as murine pneumonia. It is now widely used in many laboratories, but little has been published hitherto on normal blood values in this species needed as a baseline for evaluating experimental results. In a recent study in this laboratory, blood values were determined for fed and fasted Mystromys and compared with those of Osborne-Mendel rats; a paper is in press. Mystromys of both sexes had higher serum values of glutamic pyruvic transaminase and urea nitrogen and lower lactic dehydrogenase, alkaline phosphatase and glucose values than those of Osborne-Mendel rats. Female Mystromys had lower serum glutamic oxalacetic transaminase values than female rats. Serum aldolase values were comparable in the 2 species. The serum alkaline phosphatase was much lower in Mystromys than in rats and did not fall after fasting as it did in rats. A major portion of the serum alkaline phosphatase in Mystromys was due to intestinal alkaline phosphatase produced by the intestinal epithelium, as demonstrated in histochemical studies.

In another study on Mystromys, 17 animals were selected for study because of partial oculocutaneous albinism. This study is being carried out with the collaboration of the Ophthalmic Pathology Branch, AFIP, and includes histologic as well as electron microscopic studies of the eye of these animals. A paper on the histologic changes is nearing completion.

Radiological-Pathological Correlation: Correlation between the radiologic and gross and microscopic findings has been found highly

efficacious in giving radiologists a better understanding of the significance of various radiologic signs and in improving their diagnostic acumen. This method is used extensively by the Radiologic Pathology Branch in its exceedingly popular courses for radiology residents.

In a study with Capt. E. G. Theros, MC, USN, Chief of the Radiologic Pathology Branch, a correlation was made between the radiologic findings in bronchioloalveolar cell carcinoma and the gross and microscopic findings. Selected gross and histological specimens were used to explain the mechanisms underlying the radiographic signs. The fibroblastic reaction in the presence of this tumor resulted in the radiographic visualization of well defined streaks of density in and around these tumors in long-standing cases.

Honors and Awards:

Delegate to the House of Delegates of the College of American Pathologists for the PHS.

Member of the Executive Committee and Vice Chairman of the Section on Governmental Pathology, College of American Pathologists.

Received certificate for successfully completing Radioisotope Users Course given at the Walter Reed Army Institute of Research, June 22 to July 22, 1970.

Publications:

Highman, B., Rantanen, N. W. and Hanks, A. R.: Effect of epinephrine in oil on susceptibility of x-irradiated rats to experimental bacterial endocarditis. J. Infect. Dis. 121: 656-659, 1970.

Highman, B., Hanks, A. R. and Rantanen, N. W.: Effect of regional shielding on bacterial endocarditis in x-irradiated rats. Radiat. Res. 43: 691-697, 1970.

Theros, E. G. and Highman, B.: RPC of the Month from the AFIP - Bronchioloalveolar cell adenocarcinoma. Radiology 97: 661-668, 1970.

Streett, R. P. and Highman, B.: Blood chemistry values in normal Mystromys Albicaudatus and Osborne-Mendel rats. Lab Animal Care (in press).

Serial No. NIAMD-LEP-3
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Preparation of stained tissue sections for investigation and diagnostic purposes.

Previous Serial Number: Same

Principal Investigator: Mr. Walter Rawlings - Head, Tissue Preparation Laboratory

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 4
Professional: 0
Other: 4

Project Description:

The statistical report of this unit is shown below. In addition to preparing material for projects conducted in the Laboratory of Experimental Pathology, many other investigators received advice and service. These include: Dr. Cheever, Laboratory of Parasitic Diseases, NIAID; Drs. Rowe and Cook, Laboratory of Viral Diseases, NIAID.

	Specimens Accessioned	Stained Slides Routine	Stained Slides Special	Spare Slides	Total
Animals*	2,485	6,826			
Surgical	1,582	2,519			
Autopsy	8	66			
		9,411	5,507		
		14,918		17,449	32,367

*Source of animal material:
NIAMD 2,056
Other Institutes 429

Serial No. NIAMD-LEP-4
1. Experimental Pathology
2. Chemical Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Isolation and study of membrane proteins.

Previous Serial Number: Same

Principal Investigator: Vincent T. Marchesi

Other Investigators: Drs. Tillack, Scott, Jackson, Segrest and Rosai

Cooperating Unit: LEP-NIAMD

Man Years:

Total: 6
Professional: 3
Other: 3

Project Description:

Further characterization of the principal glycoprotein of the human red cell membrane: A single glycoprotein can be isolated from red blood cells by treating ghost membranes with lithium diiodosalicylate (Marchesi, Fed. Proc. 29: 600, 1970). The purified molecule appears homogeneous, has A, B, and MN blood group activities and the carbohydrate receptors for influenza virus, phytohemagglutinin, and wheat germ agglutinin. Trypsin hydrolysis of the protein yields two classes of glycopeptides (α and β) which have been partially purified by a combination of gel filtration and chromatography on Dowex 50-X2 and DEAE-cellulose. Both glycopeptides have a molecular weight of approximately 15,000 but differ in amino acid compositions and their mobilities on acrylamide gel electrophoresis. Both have the receptors for influenza virus and phytohemagglutinin. Cyanogen bromide cleavage of the glycoprotein yields glycopeptides and peptides containing no detectable carbohydrate. One of the latter peptides has a molecular weight of approximately 12,000 and contains the bulk of the hydrophobic amino acids of the original glycoprotein. Trypsin digestion of intact red blood cells results in the preferential release of the α glycopeptides. The β glycopeptides are released by trypsin only after the modified glycoprotein (minus α) has been isolated. These results suggest that the glycoprotein is a single polypeptide chain composed of two glycopeptide portions and a hydrophobic segment (with R. L. Jackson and J. P. Segrest).

Role of glycoproteins in membrane structure: It has been suggested that glycoproteins on the red cell surface are oriented with their carbohydrate-rich portion exposed to the exterior and hydrophobic segments embedded in the lipid of the membrane (Morawiecki, Winzler). This view is based primarily on the results of proteolytic digestion of intact red cells and on trypsin treatment of isolated glycoproteins. Direct evidence in support of this model has been obtained which relates the glycoprotein of the red cell membrane to 75 Å globular particles which are located within the lipid portion of the membrane.

The distribution of glycoproteins on the surfaces of intact red cells and ghosts was mapped by studying replicas of freeze-etched preparations by electron microscopy. Although the exposed portions of glycoprotein molecules are too small to be seen by this technique, specific sites on the molecules can be labelled by markers (influenza virus, phytohemagglutinin, or ferritin-conjugated phytohemagglutinin) which are easily recognized. The distribution of these markers indicates that the glycoproteins are dispersed uniformly over the surfaces of the cells in the same distribution as the intramembranous particles. Trypsin treatment of red cell ghosts results in a rearrangement of the intramembranous particles, and the labelled sites on the outer surface also shift to correspond to the new particle pattern.

It is not yet clear how the glycoproteins are connected to the intramembranous particles. One idea is that hydrophobic parts of the molecules interact with lipids and/or proteins, and together this complex forms the globular unit (with T.W. Tillack and R.E. Scott).

A new method for the isolation of tumor-specific antigens from human neoplasms: Carcinomas of the gastrointestinal tract have a membrane-bound glycoprotein (called carcinoembryonic or CEA antigen) which is not detectable in normal adult tissue. This tumor-specific protein can also be assayed immunologically in the sera of patients with gastrointestinal carcinomas (Thomson et al., Proc. Nat. Acad. Sci. 64: 161, 1969), thus providing a new method for the early diagnosis of cancer. The original method for the isolation of CEA antigen involves extraction of tumors with perchloric acid and fractionation of acid-soluble glycoproteins by electrophoresis and gel filtration. We have developed a new method using lithium diiodosalicylate (LIS), which has a number of advantages: LIS dissociates membranes into their macromolecular components under relatively mild conditions, and the entire glycoprotein components of the membranes can be extracted in water-soluble form without the use of perchloric acid.

A glycoprotein, extracted with LIS from human colonic tumors, has been purified by cold phenol extraction, ion exchange chromatography, gel filtration and isoelectric focusing. It is homogeneous by acrylamide gel electrophoresis and is immunologically identical with the original CEA antigen supplied by P. Gold. This technique is effective in

extracting membrane glycoproteins from other cell types and is now being used to isolate membrane proteins from other human tumors (with J. Rosai and T. W. Tillack).

Publications:

Marchesi, V. T.: Ultrastructural aspects of acute inflammation. In Sommers, S. C. (Ed.): Pathology Annual 1970. New York, New York, Appleton-Century-Crofts, 1970, pp. 343-353.

Tillack, T. W. and Marchesi, V. T.: Demonstration of the outer surface of freeze-etched red blood cell membranes. J. Cell Biol. 45:649-653, 1970.

Marchesi, V. T.: Mechanisms of blood cell migration across blood vessel walls. In Gordon, A. S. (Ed.): Regulation of Hematopoiesis. New York, New York, Appleton-Century-Crofts, 1971, pp. 943-958.

Marchesi, V. T.: Some properties of membrane glycoproteins. Monograph published by Amer. Assoc. of Path., 1971, in press.

Nicolson, G. L., V. T. Marchesi and S. J. Singer: The localization of spectrin on the inner surface of human red blood cell membranes by ferritin-conjugated antibodies. J. Cell Biol, 1971, in press.

Serial No. NIAMD-LEP-5
1. Experimental Pathology
2. Chemical Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Cell membrane ultrastructure studied by freeze-etching.

Previous Serial Number: Same

Principal Investigator: Thomas W. Tillack

Other Investigators: Drs. V. T. Marchesi, R. E. Scott, and J. Rosai.

Cooperating Units: LEP-NIAMD

Man Years:

Total: 1
Professional: 1
Others: 0

Project Description:

We have been studying the structural organization of red cell membranes by a combination of biochemical and morphological techniques. We have used freeze-etching to study the ultrastructure of membranes, since this technique exposes large faces of membrane at various levels in the membrane, and these can be examined at high resolution in the electron microscope.

Freeze-etching has demonstrated globular particles about 70 Å in diameter within the internal matrix of membranes, and we have been investigating the chemical nature and function of these particles. Treatment of red cell membranes with reagents which remove protein without breaking covalent bonds can remove about 50% of membrane protein (i.e., dialyzing membranes against EDTA, hypertonic salt solutions, etc.), but the particles were still present in the freeze-etched membranes. Phospholipase C did not remove the particles. Incubation of membranes with proteases (Pronase, trypsin) removed about 80% of the membrane protein, but the globular particles were still present, although their distribution within the membrane was altered--they were now aggregated into clumps. Lithium diiodosalicylate treatment of membranes, which extracts glycoproteins from the membranes, resulted in removal of the particles. Since glycoproteins have one end of the molecule buried in the lipid layer of the membrane, and since freeze-etching appears to split the lipid bilayer, we concluded that

the intramembranous particles might be in part a complex of the hydrophobic ends of glycoproteins with membrane lipids and perhaps other hydrophobic proteins.

We tested this hypothesis by labeling specific sites on glycoproteins, and compared the position of the label with the position of the intramembranous particles in freeze-etch preparations. We used as labels influenza virus, whose receptor site is the terminal sialic acid of glycoprotein oligosaccharide chains, and phytohemagglutinin, whose receptor site is a specific glycopeptide on membrane glycoproteins. The influenza virions and the phytohemagglutinin molecules (in some preparations conjugated to ferritin) were found to be distributed evenly over the entire surface of normal red cell membranes corresponding to the even distribution of particles within the membrane. These labels were demonstrated to localize directly over the intramembranous particles in the membranes of red cell ghosts where the intramembranous particles had been clumped by brief trypsinization. We therefore conclude that the hydrophobic tail of membrane glycoproteins, probably in combination with bound lipids and hydrophobic portions of other proteins, form the 70 Å particles seen in freeze-etched membranes.

Publications:

Tillack, T. W. and Marchesi, V. T.: Demonstration of the outer surface of freeze-etched red blood cell membranes. J. Cell Biol. 45: 649-653, 1970.

Tillack, T. W., Carter, R., and Razin, S.: Native and reformed Mycoplasma laidlawii membranes compared by freeze-etching. Biochim. Biophys. Acta 219: 123-130, 1970.

Serial No. NIAMD-LEP-6
1. Experimental Pathology
2. Chemical Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Structure of cell membranes. Biochemical and ultrastructural studies on human erythrocytes, lymphocytes and synchronized tissue culture cells.

Previous Serial Number: Same

Principal Investigator: Robert E. Scott

Other Investigators: Drs. V. T. Marchesi and T. W. Tillack

Cooperating Units: LEP-NIAMD (Drs. Marchesi and Tillack)

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

I. Our previous studies on the isolation and characterization of membrane proteins have been extended. Of principal interest have been the studies on membrane glycoproteins and their association with membrane structures demonstrated when biological specimens are examined by freeze-etching. As reported last year, there appears to be a close correlation between membrane glycoproteins and 70 Å intramembranous globular particles which are exposed by freeze cleavage. To substantiate this hypothesis we have labelled cells with specific markers that are bound to specific glycoprotein receptors on cell membranes as a means to further correlate the location of glycoprotein and that of the 70 Å membrane particles. The receptor for influenza virus on the cell membrane is sialic acid. Since all the sialic acid of human erythrocyte membranes is a part of the glycoprotein, influenza virus can be used as a specific marker for glycoprotein. Treatment of intact erythrocytes with virus show that the entire membrane is covered by influenza virus. Such cells also demonstrate a diffuse 70 Å particle distribution. However, when erythrocyte ghosts are treated so as to cause aggregation of the 70 Å intraparticles, subsequent treatment with influenza virus showed that virus was localized only over areas of clumped particles.

Similar studies have been performed using the plant lectin, PHA, that has been covalently linked to ferritin. As with influenza virus, localization

of ferritin-PHA is also closely associated with membrane particles. Since it has been shown that the receptor for PHA on erythrocyte membranes is a glycopeptide, this study suggests a relationship between membrane glycoprotein and 70 Å membrane particles.

Based on these studies it is proposed that the glycoprotein of erythrocyte membranes is associated with the particles demonstrated by freeze-etching. Furthermore, it is suggested that the actual composition of these particles consist of a hydrophobic portion of the glycoprotein in association with other membrane proteins and some tightly bound lipid. Current studies are focused at examining the possiblity that the 70 Å intramembrane particle contains all the membrane protein in a structurally organized unit.

II. The studies on the erythrocyte membrane "glycoprotein-70 Å particle" complex, suggested to us that a change in the distribution or frequency of membrane particles might be seen as a result of cell aging. As a model system, experiments were designed to study the transformation of normal human lymphocytes induced with PHA and concanavalin A. Sequential analyses of transforming cells were made by freeze-etching. Normal peripheral blood human lymphocytes contained very few intramembranous 70 Å particles (compared to erythrocytes); however, between 24 and 36 hours after stimulation with purified PHA or ConA, a rapid 300% increase in 70 Å membrane particles was evident. Studies on the ability of normal and transformed lymphocyte to bind ferritin-labelled PHA and influenza virus were performed. Although in lymphocytes the receptor for PHA appears to be a glycolipid, the specific binding of influenza virus to glycoprotein is apparent. Therefore it demonstrated the correlation between the viral binding sites and the frequency and location of 70 Å particles suggest that glycoproteins are associated with membrane particles in lymphocyte membranes. The data shows that as the number of 70 Å particles increase in transformed cells, there is a concomitant increase in the binding of influenza virus.

The process of lymphocyte transformation by plant lectins involves the stimulation of a cell that is arrested in the early postmitotic stage of the cell cycle, to begin to synthesize DNA and to eventually divide. Since changes in the membrane of transforming lymphocytes appeared just before the onset of DNA synthesis (S phase), the possibility that the membranes of other cells may naturally undergo such changes was investigated.

III. Preliminary studies on synchronized tissue culture cells have been performed. Cell samples have been taken at multiple stages of the cell cycle and samples have been studied by freeze-etching. It has been confirmed that the membranes of early G_1 cells contain few particles, but that there is a rapid increase in particle density just before the onset of the DNA synthesis (S phase). During S, G_2 and the M phase, particle distribution shows only minor fluctuations. Current studies

are designed to attempt to correlate these changes with membrane composition. Attempts at membrane fractionation are being made as are membrane labelling studies as previously described.

The use of synchronized tissue culture cells appears to be an excellent model system to study the chemical composition of membranes at intervals when freeze-etch studies suggest there are striking alterations in membrane configuration.

Publications: None.

Serial No. NIAMD-LEP-7
1. Experimental Pathology
2. Chemical Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The structure of human red cell membrane glycoproteins.

Previous Serial Number: None

Principal Investigator: Richard L. Jackson

Other Investigators: Drs. Vincent T. Marchesi and Jere P. Segrest

Cooperating Units: LEP-NIAMD (Drs. Marchesi and Segrest)

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

During the last two years this laboratory has directed its research to the study of the properties of cell surface glycoproteins. A single glycoprotein possessing blood group activity and a number of other receptors has been isolated and has been shown to be homogeneous by a number of techniques. The molecule appears to be "bipolar" with a C-terminal peptide containing little carbohydrate and a large proportion of hydrophobic amino acids.

We speculate that the glycoprotein is anchored in the membrane by hydrophobic interactions between the C-terminal peptide and the lipid. The other end of the glycoprotein is carbohydrate-rich and is exposed to the solvent.

We have directed our attention to the study of the primary structure of this glycoprotein. We have attacked the problem by both chemical and enzymic fragmentation of the glycoprotein. Chemical cleavage with cyanogen bromide has yielded 4-5 peptides plus a glycopeptide. The peptides have been purified by a combination of gel filtration and ion-exchange chromatography. One of the peptides does not have C-terminal of the native glycoprotein.

Further studies with tryptic peptides are also in progress. Proteolytic cleavage of the native molecule with trypsin yield 14-16 peptides. Three of these peptides are glycoproteins. We have purified the glycopeptides by a combination of gel filtration and ion-exchange

chromatography.

Work is currently in progress to determine the amino acid sequence of of both the cyanogen bromide fragments and the tryptic fragments.

Publications: None.

Serial No. NIAMD-LEP-8
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Histochemistry: principles, methods and applications.

Previous Serial Number: Same

Principal Investigator: Ned Feder

Other Investigators: Michael V.L. Bennett, William I. Bennett, Milton W. Brightman, Fotis C. Kafatos, Thomas S. Reese, Walter W. Stewart.

Cooperating Units: LNS-NINDS (Reese and Brightman)
Harvard University (Kafatos)
Albert Einstein Medical School (M.V.L. Bennett)

Man Years:

Total: 7
Professional: 3
Other: 4

Project Description:

I. Microperoxidase as an ultrastructural tracer.

Ultrastructural studies have been carried out with microperoxidase (MP), a heme-peptide developed as a tracer in this laboratory. After introduction of this compound into tissues or cells, its position can be readily determined with the electron microscope. The unusual feature of MP is its small size; of the ultrastructural tracers that can be used in vivo, MP has the lowest molecular weight (about 1900). Several studies have been carried out with MP, as follows.

(a) Substructure of an electrotonic synapse. The electrotonic synapse in the giant axon of the crayfish is an easily studied example of the gap junction, a common type of cell-to-cell contact. This synapse is permeable to MP injected intracellularly, but not to horseradish peroxidase. The substructure of the synapse and the route of transmission from cell to cell via the gap junction are being studied by means of MP. (With Reese and M.V.L. Bennett.)

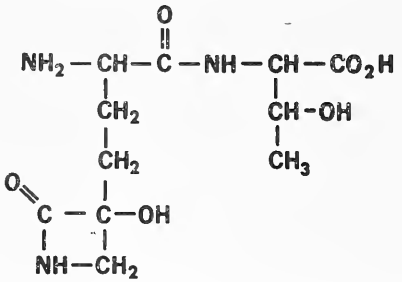
(b) Diffusion barriers in the central nervous system. Studies on the entry of MP into the periaxonal space and on the nature of the blood-

brain barrier have been continued. (See report of 1970.) (With Reese and Brightman.)

(c) Blood-testis barrier. There is a barrier to the diffusion of tracer molecules from blood across the seminiferous epithelium; in this regard the testis resembles the central nervous system (with its blood-brain barrier), but differs from most other organs, which have no such barrier. The "blood-testis barrier," as delineated by horseradish peroxidase and MP, appears to be located near the base of the seminiferous epithelium in a continuous band of tight junctions formed by Sertoli cells with each other. We have recently learned that these results are very similar to those obtained by M. Dym and D.W. Fawcett (personal communication). (With W.I. Bennett.)

II. Studies on wildfire toxin.

Wildfire toxin is produced by Pseudomonas tabaci and is highly toxic to plants. The cytotoxicity is presumed to be due to the inhibitory effect of the toxin on glutamine synthetase. The structure of the toxin, which was previously unknown, has now been elucidated. Wildfire toxin is a dipeptide yielding on hydrolysis the amino acids threonine and tabtoxinine; the toxin has the structure;



The biological activity of the toxin may be due to the β-lactam ring -- a group previously found in nature only in the penicillins, in cephalosporin C, and in the pachystermines. With the successful determinations of the toxin's structure, this project is now completed. (With Stewart.)

III. Cesium polycarboxylates for density gradient ultracentrifugation.

This study (see report of 1969) will be concluded upon completion of a report now in preparation. (With Kafatos.)

ublications:

Feder, N.: A heme-peptide as an ultrastructural tracer. J. Histochem. Cytochem. 18: 911-913, 1970.

Stewart, W. W.: Isolation and proof of structure of wildfire toxin. Nature 229: 174-178, 1971.

Serial No. NIAMD-LEP-9
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Fine structural and cytochemical studies of cell development and function.

Previous Serial Number: Same

Principal Investigator: Bruce K. Wetzel

Other Investigators: Dr. R.F. Gittes.

Cooperating Unit: University of California School of Medicine
San Diego, Calif. (Gittes)

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Chronic hypercalcemia induced in rats by parathyroidectomy has been shown to produce a more than two-fold hyperplasia of thyroid light cells as compared with normocalcemic controls. This response was nonuniform and possibly bimodal from lobe to lobe, even between contralateral lobes from the same animals; some lobes displayed a four-fold light cell hyperplasia, while other lobes contained normal numbers of light cells. In thyroid lobes showing light cell hyperplasia the mean light cell volume was increased more than 50% above control values. These increases in light cell number, volume and content of secretory vesicles as seen by electron microscopy corresponded to an increase of up to 12-fold in thyrocalcitonin content in the thyroid lobes of rats with chronic hypocalcemia, further implicating the light cell as a site of this hormone. (Wetzel and Gittes)

Comparative fine structural studies of eosinophil granulogenesis in mammals have borne out earlier suggestions (Wetzel, 1970b) that most species display a pattern resembling that of rabbits, rats, mice, guinea pigs, and humans. However, the following exceptions have been elucidated: 1) In cats all eosinophil granules, even in the earliest cells, contain dense lamellae that evidently aggregate to form the multilaminate whorls which characterize all mature eosinophil granules in this species. 2) In dogs most of the eosinophil granules are spherical and homogeneous, even in mature cells, and only a few extraordinary granules display dense crystalloids such as typify mature eosinophil granules of other mammalian species. These exceptional

cases will prove helpful in proposed autoradiographic studies of eosinophil granule formation. (Wetzel)

Publications:

Wetzel, B.K.: The fine structure and cytochemistry of developing granulocytes with special reference to the rabbit. In Gordon, A.S. (Ed.): Regulation of Hematopoiesis. New York, New York, Appleton-Century-Crofts, 1970, pp 769-817.

Wetzel, B.K.: The comparative fine structure of normal and diseased mammalian granulocytes. In Gordon, A. S. (Ed.): Regulation of Hematopoiesis. New York, New York, Appleton-Century-Crofts, 1970, pp 819-872.

Wetzel, B.K., Spicer, S.S., Dvorak, H. F., and Heppel, L.A.: On the cytochemical localization of certain phosphatases in Escherichia coli. J. Bacteriol. 104: 529-542.

Dvorak, H.F., Wetzel, B.K., and Heppel, L.A.: Biochemical and cytochemical evidence for the polar concentration of periplasmic enzymes in a "miniceil" strain of Escherichia coli. J. Bacteriol., 104: 543-548.

Wetzel, B.K., and Gittes, R.F.: Thyroid light cell hyperplasia in rats with chronic hypocalcemia. Z.Zellforsch. mikro. Anat., in press.

Serial No. NIAMD-LEP-10
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Thyroid peroxidase cytochemistry.

Previous Serial Number: Same

Principal Investigator: Lois W. Tice

Other Investigators: Dr. Seymour Wollman

Cooperating Units: LP-NCI

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Factors influencing the cytochemical localization of thyroid peroxidase, the enzyme responsible for iodination of thyroglobulin, were investigated. Activity in the lumen of the thyroid follicle was present after brief fixation and disappeared as fixation was prolonged. Occurrence of luminal staining was found to be due to the persisting of endogenous peroxide production in briefly fixed thyroid, since it could be abolished by agents which blocked peroxide production or consumed peroxide, and could be produced by mild oxidation of thyroids in which endogenous peroxide production was minimal. Mild oxidation also resulted in the appearance of membrane-associated staining, both in intracellular sites and at the apical cell border of thyroid follicular cells -- a distribution consistent with biochemical results to date. Present observations suggest that diaminobenzidine, an essential component of the cytochemical peroxidase medium, is responsible for the loss of luminal and membrane staining often observed after cytochemical incubation.

Publications:

Wolfe, S., Cage, G., Epstein, M., Tice, L., Miller, H., and Gordon, Jr., R.S.: Metabolic studies of isolated human eccrine sweat glands. J. clin. Invest. 49: 1880-1884, 1970.

Serial No. NIAMD-LEP-11
1. Experimental Pathology
2. Rheumatic Diseases
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Pathogenesis of experimental arthritis and pathology of rheumatism.

Previous Serial Number: Same

Principal Investigator: Leon Sokoloff, M.D.

Other Investigator: Ralph E. Marcus, M.D.

Cooperating Units: NICHD (William H. Simon, M.D.)
NIDR (Don L. Layman, Ph. D.)
NCI (William G. Banfield, M.D.)

Man Years:

Total: 5
Professional: 2
Other: 3

Project Description:

1) Investigation of factors influencing development of degenerative joint disease in experimental animals and men.

2) Extending descriptive pathology of human rheumatic disease.

Methods:

1) Articular chondrocytes are cultured in vitro and synthesis of specific cell products is measured.

2) Scale effects on several biomechanical parameters of animal joints are investigated.

Major Findings:

1) A heat-labile anterior pituitary factor exerts a strong mitogenic action on articular chondrocytes in secondary monolayer culture. It was found in NIH bovine and ovine TSH as well as LH but not in more purified (Condliffe-Bates, Pierce) preparations of TSH. The response, measured by the DNA content of the cell pellet, was dose-dependent and evident at a concentration of 1μg/ml culture medium.

The effect was fairly selective for chondrocytes, rabbit and human, and displayed to little or no degree by skin fibroblasts and other cell types studied. FSH had a smaller effect while GH, prolactin, ACTH and a preparation having high exophthalmos-producing activity were relatively ineffective. Of 15 other hormone preparations examined, only crude human chorionic gonadotropin gave a comparable mitogenic response. Insulin, 0.1 u/ml, had a consistent but small effect. Secretion of macromolecular radiosulfate into the medium by the stimulated cells was markedly reduced.

2) The wear of articular cartilage (human patellar and canine humeral head) was studied in vitro with a rotating steel abrader. With saline lubrication at 37°C, the cartilage was more resistant to abrasion than neoprene rubber (shore duometer 50), oak and balsa wood, but less than that of a series of plastics (nylon, high and low density polyethylenes, polycarbonate and polytetrafluoroethylene). Synovial fluid had a distinct wear-protecting effect; this was eliminated by digestion of the mucin with trypsin but not testicular hyaluronidase. Fibrillated cartilage was abraded more readily than intact tissue, but age otherwise had no effect. It is concluded that surface friction as well as intrinsic properties of the cartilage (strength and stiffness) are major determinants of its wear properties.

3) The deformability of articular cartilage was studied in vitro in 64 bovine, canine and human joints with an indenting compressometer. Two factors contributed to the depth of the indentation: the thickness of the cartilage and its intrinsic elasticity. Within a given species, there was in general a consistent curvilinear relationship between the magnitude of the deformation and the thickness of the articular cartilage under a given level of static loading (usually 106 pounds per square inch). Although it was not possible to establish a satisfactory Young's modulus, differences in intrinsic elasticity were demonstrated by variations in the indentations produced in cartilages of comparable thickness, when subjected to the same compressive stress. These species differences corresponded roughly to variations in the water content of the articular cartilages (bovine, 87%; canine, 80; human, 79). The data are consistent with the hypothesis that deformability of articular cartilage serves to increase the congruence of joints and thereby reduces the stresses on their surfaces. A frequently postulated senescent thinning of articular cartilage received no support from measurements of 28 intact human patellas through the fifth decade of life.

4) A cryoprobe (-20 to -80°C) was applied for 10-60 seconds to cause necrosis of metatarsal head cartilages of eleven rabbits. Segmental loss of nuclear staining was noted at one week and depletion of chondroitin sulfate (toluidine blue metachromasia and staining with safranin O) a few days later. No degenerative joint disease was evident up to six months. The latter finding suggests that, in

addition to chondrocyte death, externally imposed stresses are necessary to disrupt the collagenous 'skeleton' and initiate the changes of degenerative joint disease.

5) The anatomic findings in previous projects have been extended.

A. Histologic studies performed between April 26, 1970 and April 6, 1971, in addition to 7 necropsies on patients with rheumatic diseases, include:

Joint	Skin	Muscle	Other
23	20	10	49

B. A case of what clinically is considered to be aseptic necrosis of bone in a steroid-treated patient with lupus erythematosus, proved anatomically to be an infraction of a porotic bone cortex. Other instructive cases studied were multicentric lipohistiocytosis (1); lymphomatoid pulmonary involvement in Wegener's syndrome (1).

Proposed Course:

The main goal of the project over the last 18 years has been an understanding of the pathogenesis of degenerative joint disease, and centers about the interplay between aging and mechanical use in the wear and tear of joints. In vitro culture will be the principal focus of research. The cartilage growth factor noted above will be characterized further. The following studies already under way should be completed in another year: the behavior of articular chondrocytes under anaerobic conditions; the characterization of the molecular species of collagen produced by chondrocytes in culture; the electronmicroscopic nature of amianthoid degeneration of cartilage. A visiting fellow will be working on the mucopolysaccharide products of the cultured cells.

Publications:

Green, W.T., Jr.: Behavior of articular chondrocytes in cell culture. Clin. Orthop. 75: 248-260, 1971.

Green, W.T., Jr., Martin, G.N., Eanes, E.D. and Sokoloff, L.: Microradiographic study of the calcified layer of articular cartilage. Arch. Path. 90: 151-158, 1970.

McCutchen, C.W.: The trout tail fin: A self-cambering hydrofoil. J. Biomechan. 3: 271-281, 1970.

McCutchen, C.W.: Surface films compacted by moving water: Demarcation lines reveal film edges. Science 170: 61-64, 1970.

McCutchen, C.W.: Apodization of astronomical telescopes: Effect of atmospheric turbulence. J. Opt. Soc. Amer. 60: 1534-1535, 1970.

Simon, W.H.: Scale effects in animal joints. I. Articular cartilage thickness and compressive stress. Arthritis Rheum. 13: 244-256, 1970.

Simon, W.H.: Scale effects in animal joints. II. Thickness and elasticity in the deformability of articular cartilage. Arthritis Rheum. In press.

Simon, W.H.: Wear properties of articular cartilage in vitro. J. Biomechan. In press.

Simon, W.H., and Garman, R.A.: Simian bone disease: Unrecognized rickets in rhesus monkeys. Clin. Orthop. 73: 232-240, 1970.

Simon, W.H., and Green, W.T., Jr.: Experimental production of cartilage necrosis by cold injury: failure to cause degenerative joint disease. Amer. J. Path. In press.

Sokoloff, L., Malemud, C.J., and Green, W.T., Jr.: Sulfate incorporation by articular chondrocytes in monolayer culture. Arthritis Rheum. 13: 118-124, 1970.

Steinberg, A.D., Green, W.T., Jr., and Talal, N.: Thrombotic thrombocytopenic purpura and Sjögren's syndrome. J.A.M.A. 215: 757-761, 1971.

Wilson, J.T., and Sokoloff, L.: Epidermoid cysts simulating rheumatoid nodules in the olecranon region. J.A.M.A. 214: 593-595, 1970.

Serial No. NIAMD-LEP-12
1. Experimental Pathology
2. Histochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Protein and Proteolytic Enzyme Interrelationships in Normal and Disease States.

Previous Serial Number: NIAMD-LEP-13

Principal Investigator: George G. Glenner, M.D.

Other Investigators: C. Isersky, Ph.D., Visiting Associate (July 1, 1968-July 1, 1971), D. Page, M.D., Research Associate (July 1, 1969 - July 1, 1971)

Cooperating Units: M. Harada, Ph.D. (Aichi-Gakuin University, Nagoya, Japan)
H. Bladen, Ph.D. (NIDR-LHP)
D. Eanes, Ph.D. (NIDR-LHP)
P. Cuatrecasas, M.D. (Johns Hopkins Medical School, Baltimore, Maryland; formerly NIAMD-LEB)
H. R. Keiser, M.D. (NHI-LET)
W. Terry, M.D. (NCI-I)

Man Years: (computed for the 12 month period)

Total:	3
Professional:	2
Other:	1

Project Description:

Human Amyloidosis

The major protein of amyloid fibril concentrates of ten tissues obtained from six patients was fractionated by sequential gel filtration on Sepharose 4 B and Sephadex G-100 columns using 5 M guanidine-HCl in 1 N acetic acid with removal of over 28% of minor constituents. The major amyloid protein from each individual differed from that of the others in molecular weight, amino acid composition and peptide map profile. The major protein from the liver and spleen of the same individual, however, were identical. A serum component having an immunologic relationship to the patients' major amyloid protein was detected migrating to the γ-globulin region on immunoelectrophoresis. In view of the marked chemical similarity of amyloid protein and immunoglobulin protein, e.g.

unreactive or Asx N-terminal groups, constant tryptophan content, heterogeneity between individuals and homogeneity within the individual, and the molecular weight ranges of amyloid fibril protein found, the possibility that amyloid might be derived from the N-terminal variable segment of the light or heavy chain of immunoglobulin proteins (e.g. the Fd fragment) was considered.

In order to test this postulate directly and conclusively, the amino-terminal amino acid sequencing of one of the purified amyloid proteins was undertaken using an automatic amino acid sequencer, Beckman Model 890, following the method of Edman and Begg. The sequence of this amyloid protein was found to be

Asp-Ile-Gln-Met-Thr-Gln-Ser

This amino-terminal amino acid sequence categorized this amyloid protein as belonging to a subgroup of immunoglobulin light polypeptide chains similar to kappa I. Further sequencing of this protein to position 36 and of another amyloid protein to position 35 revealed both to be homologous to a previously sequenced Bence-Jones κ-protein of variable region subgroup $V_{\kappa I}$. These amyloid proteins are, therefore, portions of κ-type light chains of the $V_{\kappa I}$ variable region subgroup. The molecular weight of an intact light polypeptide chain is approximately 22,500. The molecular weights of 7,500 and 18,300 for these amyloid proteins, as determined by both SDS polyacrylamide gel electrophoresis and calibrated Sephadex G-100 columns, indicate that these proteins are not complete light chains. Since both proteins start with the appropriate amino-terminal sequence, it is possible that these represent light chain fragments with varying amounts of the constant portion degraded. Alternatively, amyloid may consist of light polypeptide chains having internal deletions of varying lengths affecting variable regions, constant regions, or both, such as those reported in "heavy chain disease" proteins.

The losses on purification and the yield on sequence analysis (when compared with known homogeneous light chains) indicate that the proteins sequenced are the major components of amyloid and are not minor contaminants. Since only 30 percent of the protein content of the fractionated amyloid fibril concentrate is lost in purification and since sequence analysis and disc gel electrophoresis give evidence of the presence of a single monomeric component in the final preparation, the possibility that non-specifically absorbed homogeneous immunoglobulins accounted for 70 percent of the amyloid fibril concentrate would appear highly unlikely. The major protein component of these amyloid preparations is, therefore, a portion of an immunoglobulin light polypeptide chain and amyloidosis is, therefore, a disease caused by the deposition in tissues of an amino-terminal fragment of the variable region of the light chain of homogeneous immunoglobulins by a mechanism

presently under investigation.

The above findings not only describe the nature of amyloid, but provide a method of isolation of a homogeneous portion or fragment of immunoglobulins believed to be important in antigen-binding, one of the first steps in the bodily defense mechanism for removing bacteria and other foreign proteins. This leads to the possibility of a better understanding of the mechanism of this crucial step in the body's defense against disease. In addition these findings indicate that amyloidosis is the final common pathway of what may be a variety of synthetic or degradative disorders of immunoglobulin metabolism. It is one of the few diseases of immunoglobulin metabolism in which the nature of the lethal component has been defined on a molecular basis.

Honors and Awards: President Elect, Histochemical Society, 1971-72.

Publications:

Nagatsu, I., Nagatsu, T., Yamamoto, T., Glenner, G. G., and Mehl, J. W.: Purification of aminopeptidase A in human serum and degradation of angiotensin II by the purified enzyme. Biochim. biophys. Acta 198: 255-270, 1970.

Glenner, G., Harada, M., Isersky, C., Cuatrecasas, P., Page, D., and Keiser, H.: Human amyloid protein: Diversity and uniformity. Biochem. Biophys. Res. Comm. 41: 1013-1019, 1970.

Glenner, G. G., Harbaugh, J., Ohms, J. I., Harada, M., and Cuatrecasas, P.: An amyloid protein: The amino-terminal variable fragment of an immunoglobulin light chain. Biochem. Biophys. Res. Comm. 41: 1287-1289, 1970.

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Harada, M., Isersky, C., Cuatrecasas, P., Page, D., Bladen, H. A., Eanes, E. D., Keiser, H. R., and Glenner, G. G.: Human amyloid protein: Chemical variability and homogeneity. J. Histochem. Cytochem. 19: 1-15, 1971.

Glenner, G. G., Page, D., Isersky, C., Harada, M., Cuatrecasas, P., Eanes, E. D., DeLellis, R. A., Bladen, H. A., and Keiser, H. R.: Murine amyloid fibril protein: Isolation, purification and characterization. J. Histochem. Cytochem. 19: 16-28, 1971.

Serial No. NIAMD-LEP-13
1. Experimental Pathology
2. Histochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Protein-protein Interactions and Immune Processes in Normal and Disease States.

Previous Serial Number: NIAMD-LEP-14

Principal Investigator: J. Sri Ram, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years: (computed for the 12 month period)

Total:	1
Professional:	1
Other:	0

Project Description:

Glutathione - Immunochemical and Other Studies

The importance of glutathione in normal and disease states has been well established. This peptide seems to occur in a free state as well as linked to various proteins of cellular origin including membrane proteins. This and the absence of suitable, specific histochemical analytic methods for the detection of glutathione prompted an attempt at obtaining antibodies specifically reacting with glutathione moiety.

Studies directed at obtaining antibodies specific for glutathione were continued. A variety of polymers of glutathione were prepared under different experimental conditions employing either water-soluble carbodiimides or p,p'-difluoro-m,m'-dinitrodiphenylsulfone. In addition, glutathione was covalently linked to three proteins (rabbit serum albumin, bovine serum albumin and lysozyme, respectively) by means of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. Control proteins for the above preparations included: protein treated with glutathione in the absence of carbodiimide and protein treated with carbodiimide alone under identical experimental conditions.

Rabbits were immunized with several of the above preparations, namely, a) glutathione polymer prepared through carbodiimide reaction, b) polymer prepared by means of the sulfone reagent, c) rabbit

albumin-glutathione conjugate, d) bovine albumin-glutathione conjugate and e) bovine albumin treated with carbodiimide. In each case the "antigen" was incorporated in complete Freund's adjuvant and injected into rabbits repeatedly as desired. At various intervals during the immunization, the animals were bled and the sera were tested for homologous and heterologous reactions employing various GSH derivatives and controls. The results of the various studies were as follows:

1. Immunization of rabbits with either of the glutathione polymers has so far not resulted in the elicitation of antibodies specific for glutathione moiety. The techniques employed for the detection of antibodies included gel diffusion in agar, complement fixation and the more sensitive passive cutaneous anaphylaxis test.

2. Likewise, immunization of rabbits with conjugate of glutathione with rabbit albumin proved ineffective. In this case, it was hoped that since the carrier protein is homologous rabbit protein, any antibodies formed would be directed almost exclusively against the "hapten" glutathione, possibly including some adjacent amino acid residues of the carrier. No reaction was observed when the resulting sera were tested against the homologous antigen or with conjugates of glutathione with unrelated proteins such as lysozyme.

3. When the carrier protein was changed from the homologous (rabbit) to a foreign origin, more encouraging results were obtained. When rabbits were immunized with a glutathione conjugate of bovine albumin, the animals responded with the elicitation of at least three types of antibodies, as tested by careful gel diffusion analysis with the various conjugates and control proteins. The multiple specificity of the antibodies was distributed as follows: a) against native bovine albumin, b) against carbodiimide modified bovine albumin and c) against what appears to be glutathione moiety. The last mentioned appears to be a small proportion of the total antibody. Attempts are underway to obtain stronger antisera by prolonged immunization. After establishing unequivocally the specificity of the last group of antibodies by techniques involving reciprocal cross reactions in gel diffusion, inhibition studies with oxidized glutathione, polymerized glutathione, etc., it would be of interest to examine the suitability of these antibodies in histochemical localization of protein-bound glutathione and perhaps of free peptide.

Experimental Pathology - Enzyme Aberrations

In last year's report, initiation of a novel series of experiments involving attempted induction of enzyme deficiency by immunological techniques (active immunization with the enzyme or passive transfer of antibodies obtained in a different species) was reported. An uncharted course of this type necessarily involves investigation of more than one

enzyme and multiple species of animals before the feasibility of the approach can be evaluated. Furthermore, the study should also cover enzymes whose deficiencies are known to cause certain well defined diseases as well as enzymes which are vital for normal function and whose decrease may consequently be expected to lead to disease.

The initial experiment in this approach was necessarily limited to one enzyme and one species, namely creatine phosphokinase and rabbit. Active immunization was the first method of choice. Rabbits were immunized with the enzyme of rabbit origin, incorporated in complete Freund's adjuvant. Prolonged immunization has so far yielded negative results. No apparent decrease in the enzyme activity was observed, nor were any signs of sensitization to the antigen or clinical difficulties noted. In this approach, it was hoped that by active immunization with the homologous antigen, enzyme deficiency might occur through an "auto-immune" type of mechanism. The alternate approach of passive transfer of antibodies obtained in a heterologous species are now underway. These studies will be expanded to other enzymes and other species of animals.

This approach of in vivo use of specific antisera is being extended to peptide hormones. In an experiment underway, in collaboration with Dr. P. Kohler of NCI, the effect of treatment with anti-chorionic gonadotropin on the growth of the tumor producing this hormone (choriocarcinoma) is being investigated. Progress in this study so far includes obtaining specific rabbit sera to the hormone and serial transplantation of the tumor in hamsters to obtain sufficient number of animals for the experiment designed.

Honors and Awards: J. Sri Ram was invited to serve as Visiting Professor at the Department of Biochemistry, Indian Institute of Science, Bangalore, India.

Publication:

Ram, J. S.: Aberrant enzymes. Biochemical Reviews 42: 1971.

Serial No. NIAMD-LEP-14
1. Experimental Pathology
2. Histochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Experimental Models of Human Disease of Protein Metabolism.

Previous Serial Number: None

Principal Investigator: David L. Page, M.D.

Other Investigators: G. G. Glenner, M.D. and C. Isersky, Ph.D., Visiting Associate (July 1, 1968-July 1, 1971)

Cooperating Units: H. Bladen, Ph.D. (NIDR-LHP)
D. Eanes, Ph.D. (NIDR-LHP)
P. Cuatrecasas, M.D. (Johns Hopkins Medical School, Baltimore, Maryland; formerly NIAMD-LEB)
H. R. Keiser, M.D. (NHI-LET)
M. Potter, M.D. (NCI-LBGY)

Man Years: (computed for the 12 month period)

Total:	2
Professional:	1
Other:	1

Project Description:

Experimental Amyloidosis

Murine amyloid has been produced by four different induction methods (Mycobacterium butyricum, casein, casein plus Freund's adjuvant and endotoxin-induced mouse amyloidosis) in several strains and obtained from mice with "spontaneous" amyloidosis. The amyloid fibrils have been concentrated from spleen and liver. Electron microscopy of all of these preparations reveals the amyloid fibril to be 100 Å in width and composed of two parallel filaments, each measuring 35-40 Å in width and having the appearance of a twisted ribbon. X-ray diffraction of all preparations reveals a "backbone" spacing at 4.75 Å and a "side chain" spacing at 11 Å indicating a β-pleated sheet" structure. Identification of the major protein component of amyloid fibril concentrates was made by combined use of sodium dodecyl sulfate polyacrylamide disc electrophoresis, labeling of the protein with ^{3}H-tryptophan and Sepharose 4 B gel filtration. Purification of the amyloid protein from spontaneous amyloidosis liver was accomplished by sequential gel filtration with 5 M guanidine in 1 N acetic acid on Sepharose 4 B and

Sephadex G-100 or G-75 columns. The material is a unique protein with a molecular weight of 7200, a high content of dicarboxylic and short chain amino acids, a significant amount of tryptophan and an unreactive amino-terminal amino acid. Murine amyloid protein, therefore, has striking similarities to many human amyloid protein preparations. It differs from the human proteins in the similarity of molecular weights of different preparations. Since human amyloid protein has been found to be derived from the amino-terminal variable fragment of the light polypeptide chain of immunoglobulins, an investigation of murine amyloid fibril protein, to determine if it has a similar origin, has been undertaken. Preliminary evidence indicates that one of the murine amyloid proteins is derived from a λ-light chain. Evidence from this study indicates that the experimental animal model is a valid counterpart of the human disease and will be of value in defining the pathogenesis of amyloidosis.

A detailed study of "spontaneous" amyloidosis in mice has revealed that this process, previously attributed to aging factors, is the direct result of chronic inflammation consequent to aggressive behavior of male mice in a colony. This study emphasizes the necessity of investigating all cases of so-called idiopathic or "primary" amyloidosis in humans in order to determine if an underlying infectious process or metabolic derangement is a predisposing factor for the development of amyloidosis in any case of this disease.

Honors and Awards: None

Publications:

Glenner, G., Harada, M., Isersky, C., Cuatrecasas, P., Page, D., and Keiser, H.: Human amyloid protein: Diversity and uniformity. Biochem. Biophys. Res. Comm. 41: 1013-1019, 1970.

Harada, M., Isersky, C., Cuatrecasas, P., Page, D., Bladen, H. A., Eanes, E. D., Keiser, H. R., and Glenner, G. G.: Human amyloid protein: Chemical variability and homogeneity. J. Histochem. Cytochem. 19: 1-15, 1971.

Glenner, G. G., Page, D., Isersky, C., Harada, M., Cuatrecasas, P., Eanes, E. D., DeLellis, R. A., Bladen, H. A., and Keiser, H. R.: Murine amyloid fibril protein: Isolation, purification and characterization. J. Histochem. Cytochem. 19: 16-28, 1971.

Serial No. NIAMD-LEP-15
1. Experimental Pathology
2. Histochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Cytogenetics

Previous Serial Number: NIAMD-LEP-16

Principal Investigator: Dr. J. H. Tjio

Other Investigators: Dr. B. White (NIAMD-LEP)

Cooperating Units: Dr. W. Pahnke (Maryland Psychiatric Research Center
Dr. E. Driscall (NIDR-OMS)
Dr. J. Robbins (NCI-D)

Man Years:

Total:	3
Professional:	1
Other:	2

Project Description:

Cytogenetic Studies on the Effect of LSD

We are continuing a double-blind, controlled research project on the effects of LSD on human peripheral blood chromosomes. Our current series of patients, who are receiving LSD as part of their psychiatric treatment at Spring Grove State Hospital in Baltimore, has been expanded to include 25 persons given DPT (another hallucinogen). As before, two pre-treatment and two post-treatment blood samples are drawn on different days from each patient. Although the coded samples cannot be identified until the study is complete, we still have found no cultures with a chromosome aberration rate greatly in excess of rates considered normal for the general population. This trend again supports our previous work.

Studies Using the Mixed Leukocyte Reaction to Examine Cellular Immunity in vitro

Blastogenesis occurs when peripheral blood leukocytes of unrelated individuals are cultured together. This "mixed leukocyte reaction" is a function of the degree of genetic compatibility between two people's cells. It has been determined that there are pairs of siblings whose

cells give no detectable reaction together but who are markedly different in their response to some antigen. Experiments are planned in which cultures containing various combinations of lymphocytes, purified in nylon-fiber columns, and glass-adherent cells (mostly macrophages) from two such donors are incubated in the presence of the appropriate antigen. The origin of the responding cells can be determined by chromosome analysis if the donors are of different sexes; this should aid in the identification of the roles of the cells involved in the immune response.

The first series of these cultures was unsatisfactory, as the reactions of the two donors to the antigenic challenge were not sufficiently different. The project will continue when more suitable individuals have been found.

Delayed Fertilization and the Chromosome Constitution of Mouse Embryos

This project was initiated to observe the rise in the number of triploid mouse embryos reported to occur following fertilization of the eggs several hours after ovulation. Unfortunately, the study is still in its preliminary stages, as chromosome preparations of the pre-implantation blastocysts have not yet been consistently satisfactory.

Cytogenetic Studies on T1Wh Translocation Mouse Strain

A. Evaluation of Fertility: Reduction of litter size of T1Wh homozygotes with inbreeding has continued. When the strain was first developed, litter size at birth was 7.1. Average size of 35 litters of F 13 and 14 during the past year was 5.9. The inbreeding program is now in generation 15.

B. Meiotic and other Studies of Mouse Hybrids: Meiotic studies of hybrids of T1Wh and other strains with Robertsonian fusion translocations (T1Ald, T163) are in progress. These indicate that the translocations T1Wh and T163 have a single small chromosome in common, but that the chromosomes involved in the T1Ald translocation are not common to T1Wh or T163. Attempts are now being made to further reduce the mouse chromosome number from 38 to 36 by breeding together hybrids carrying both T1Wh and T1Ald chromosomes. If animals homozygous for these 2 translocations are obtained by means of these F_1 crosses or backcrosses to the parental strains, they will be used to initiate a new mouse strain carrying 36 chromosomes. Chromosome studies of mouse embryos and fetuses from crosses of the various strains are also in progress. These are part of an overall study of the segregation of hybrid translocation chromosomes and its relationship to frequency of aneuploidy among hybrid offspring.

C. Autoradiographic Studies: Preparations of spleen cells from T1Wh homozygotes and normal mice did not reveal any distinctive labeling pattern of the translocation chromosomes or other mouse autosomes. Labeling of normal and translocation animals was diffuse and non-specific. Labeling studies of the other translocation strains may be attempted in the future.

D. Transfer of Cells Across the Mouse Placenta: Chromosome preparations of fetal and maternal tissues at various stages of gestation and after delivery, utilizing the T1Wh chromosome as a marker to differentiate maternal from fetal mitoses, are complete. Chromosome preparations of various tissues of 44 offspring of 7 pregnancies did not reveal presence of maternal mitoses after screening of 14,396 cells. Screening of 5,902 maternal mitoses from lung, marrow, and spleen of 9 pregnant and post-partum mice revealed no fetal cells. These negative findings are consistent with the majority of studies now reported in the literature.

Patient Studies

Patients with a variety of disorders from the inpatient service of NIAMD were karyotyped during the past year. All were chromosomally normal except for a 29 year old male with diffuse lymphedema and normal intelligence. Several peripheral blood cultures indicated that approximately 12% of his cells were missing a small arocentric chromosome; the remaining cells were normal. The Y chromosome appeared to be present in the abnormal cells, and radioautographic studies were consistent with this. A direct marrow study showed a similar proportion of abnormal cells, and testicular biopsy revealed only chromosomally. normal primary spermatocytes. Since mosaicism for monosomy G is usually associated with mental retardation, dysmorphic features, and failure to thrive in infancy, further studies of this patient with mosaicism and his family are planned. These include staining of the patient's chromosome preparations with quinacrine mustard in order to determine with greater accuracy the specific chromosome involved. This method is reported to be useful for differentiating the Y chromosome from the small autosomes.

Cytogenetic Studies on the Effect of Diazepam (Valium) on Human Chromosomes

During the coming year, investigators of the Anesthesiology Section, OMS, NIDR will be giving normal healthy patients Valium intravenously (IV) while conducting a study comparing the effectiveness of that agent and Nitrous Oxide for psychosedation in oral surgery. Analysis of conflicting reports of chromosome studies in the literature indicate the drug does not produce chromosome damage in vitro or when given to humans orally. However, further studies are indicated because the chromosome effects have not been studied after IV administration. Also, the drug (solubilized in a complex vehicle) is being used with

increasing frequency by the IV route by physicians for sedation, muscle relaxation, and for seizure control.

Our laboratory will receive 5 blood samples from approximately 20 patients in the Valium study; samples before and after the drug is given will be analyzed for chromosome damage. All analyses will be done blindly. Results of this initial study should determine if further analyses are necessary.

Honors and Awards: None

Publications:

Whang-Peng, J., Freireich, E. J., Oppenheim, J. J., Frei, E. III, and Tjio, Joe-Hin: Cytogenetic studies in 45 patients with acute lymphocytic leukemia. J. nat. Cancer Inst. 42: 881-897, 1969.

Kamada, Nanao, Brecher, George, and Tjio, Joe-Hin: In vitro effects of chlorpromazine and meprobamate on blast transformation and chromosomes. Proc. Soc. exp. Biol. 136: 210-214, 1971.

Serial No. NIAMD-LEP-16
1. Experimental Pathology
2. Histochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of the Relationship of Chromosomal Abnormalities and Certain Viral Infections in Mice.

Previous Serial Number: NIAMD-LEP-17

Principal Investigator: Dr. Beverly White

Other Investigator: Dr. Lon White (NICHD-DB)

Cooperating Units: Dr. George Nemo (NINDS-CFR)

Man Years: (computed for the 12 month period)

Total:	1
Professional:	1
Other:	0

Project Description:

Relationship of Viral DNA to Mouse Chromosomes

Preparations of MVM (minute virus of mice) labeled with H^3-thymidine have been added to cultures of normal mouse spleen cells in order to study the possible association of the viral DNA with host chromosomes. Blind analysis of autoradiographic preparations of these cultures and their controls exposed for 3 and 24 hours before harvest was done. The intensity of labeling (number of grains per metaphase localized over chromosomes) was similar in virus and control groups, and background was low in both cases. Position of grains (end or middle of chromatid, or at centromere) was observed in both groups, and while there were differences in grain distribution between control and MVM preparations, further studies using a higher multiplicity of virus will be necessary to evaluate these. The previous study utilized a ratio of 5 viruses per cell.

Alternate tissue culture methods to grow higher titers of MVM are now being evaluated by the cooperating units, so that greater quantities of labeled virus may be prepared.

Search for Viral Antigens in Germ Cells

Evaluation of gonadal tissues for the presence of viral antigen (MVM) using fluorescent antibody techniques has not been initiated. However, studies of meiotic chromosomes of primary and secondary spermatocytes of adult male mice infected with MVM 3, 6, and 12 weeks before sacrifice have been completed. Blind analysis of virus-infected and control animals showed no specific abnormalities in either group. Other studies, including analysis of meiotic cells of animals infected while in utero, will be initiated in the future.

Honors and Awards: None

Publications: None

ANNUAL REPORT
LABORATORY OF CHEMICAL BIOLOGY
NIAMD

The Laboratory of Chemical Biology has continued its investigations of macromolecular systems with special interest in the relationships between structure and function in proteins and regulation of protein biosynthesis.

I. Chemical, functional and structural studies of the extra-cellular nuclease from Staphylococcus aureus

A. Structure-function relationships in protein derivatives prepared by proteolysis

In previous years a number of studies have been carried out (Taniuchi, Anfinsen et al.) which indicate that almost the entire amino acid sequence of nuclease is required to furnish the information that determines a stable, unique and functional structure. These conclusions were arrived at by an examination of various complementing systems of fragments which, by themselves, are inactive and more or less structureless in solution but which reconstitute functional, three-dimensional complexes when allowed to interact in a non-covalent way. Nuclease itself contains 149 amino acids. Two complexing systems that have been studied involve, in the one case, residues (6-49) plus (50-149) and, in the second case, residues (1-126) and residues (99-149). In the latter case, the redundant overlapping portion of the (99-149) fragment could be removed by trypsin digestion in the presence of the stabilizing ligands, calcium ions and thymidine diphosphate.

The system /(6-49) + (50-149)/ has now been employed to study the kinetics of association and dissociation of this noncovalent complex as a function of temperature. The fragment 50-149) was mildly acetylated with radioactive acetic anhydride. This material was then allowed to form, with (6-49), the active complex that normally results upon mixing. The addition of unlabelled (6-49) to this mixture, followed by a chromatographic separation of the complex and excess, free (6-49), made it possible to estimate the rate of exchange of the unlabelled fragment with the labelled portion of the complex. As might be expected, the rate of exchange increased markedly with temperature. This approach is now being further explored in connection with the determination of factors involved in the stability of these complexes and with the effect of synthetic variations in the sequence of the (6-49) fragment. The results should ultimately be of considerable use in understanding the problem of protein folding, and the nature of the cooperative forces in protein stabilization.

Further work on the nature of the information required to determine three-dimensional protein structure was carried out on an inactive derivative of RNase-A which lacks the terminal 4 amino acid residues (des-(119-124)-RNase). This derivative is unable to reconstitute the correct 4 disulfide bonds after these have been converted to 8 SH groups by mercaptoethanol reduction. It was shown previously that the shortened derivative could be converted to a "scrambled form," which possessed an almost random set of disulfide bonds joining the 8 half-cystine residues, when this RNase derivative was exposed

to a disulfide interchange enzyme isolated from microsomes. When, however, a peptide fragment of RNase consisting of residues (105-124) was added to the mixture of disulfide enzyme and the incorrectly oxidized, scrambled des-(119-124)-RNase, the resulting interchange that took place produced an active complex containing the proper pairing of half-cystine residues. Thus, in the case of this protein system as well as staphylococcal nuclease, it would appear that essentially all of the information in the native chain is required to determine the unique, three-dimensional structure.

Since the ultimate technique for determining, in a precise way, the structural properties of a protein is X-ray crystallography, the complementing systems of fragments mentioned above are being investigated with regard to their capacity for crystalization and ultimate high resolution structure determination. The two fragments of staphylococcal nuclease, Nuclease-T-(6-48)(residues 6 to 48) and Nucleast-T-(50-149)(residues 50 to 149) were prepared by limited digestion of nuclease with trypsin in the presence of thymidine-3',5'-diphosphate (pdTp) and Ca^{++} and purified as described in previous reports. Nuclease-T' was reconstituted by mixing of Nuclease-T-(6-48) and Nuclease-(50-149) at approximately equimolar ratio. Unbound, excess fragments were removed by digestion with trypsin in the presence of pdTp and Ca^{++}. Nuclease-T' ((6-48)+(50-149)) was purified by phosphocellulose column chromatography and was crystallized in 0.0105 M potassium phosphate buffer, pH 8.3 with 38% 2-methyl-2,4-pentanediol in the presence of pdTp and Ca^{++}, at 4°. The crystals have the form of fine needles and are very unstable at 25°. (Taniuchi, Andria, Bohnert)

B. Structure-function studies on staphylococcal nuclease by the synthesis of biologically active polypeptides

Synthesis by the solid phase method of Merrifield has been continued in a number of directions in a broad investigation of the structural basis of the enzyme activity of nuclease and, more recently, in experiments designed to help elucidate the forces that lead to nucleation of portions of the amino acid sequence as an initial event of the folding of the chain. During the study of the enzymic properties of the natural and semi-synthetic complexes formed by the fragment (6-47) and (49-149), a small but reproducible enhancement of enzymic activity by calcium and thymidine diphosphate has been observed. An even more dramatic effect occurs with the mixture of nuclease-(49-149) with some chemically modified nuclease-(6-48) forms and several analogues of synthetic-(6-47), especially with analogues which generate low-activity complexes with the native fragment in the absence of ligands. As a result of such studies, the structure-stabilizing involvement of several nuclease residues, including glutamic acid residue 10, proline 11, alanine 12, isoleucine 18, and tyrosine 27 has been described. All of these results can be rationalized with the atomic model of staphylococcal nuclease obtained by X-ray crystallographic studies at M.I.T. (Chaiken, Sanchez)

A number of systematic studies have been undertaken to attempt to improve the efficiency and dependability of the Merrifield method. It has been observed that the major part of the several reactions involved in a single cycle of chain elongation has occurred in the first few minutes and that the very long times generally employed in the solid phase technique only add a few more

percent to the completeness of coupling and deprotection steps. Our current approach is based on the selection of only the very rapid, initial part of each reaction and with the irreversible blocking of all residual unreacted groups. In this way, we hope to concentrate the method on the population of growing polypeptide chains that are most available, sterically speaking. In principle, it should be possible to snythesize a protein chain the size of nuclease in 2 or 3 days and an aim of the laboratory during the coming year will be to achieve this kind of result. Using currently available methods, however, the synthesis of a long fragment of nuclease has been under particularly active attack. This fragment involves residues (99-149) and, as mentioned above, complements with (1-126) to generate an active complex. The (99-149) portion contributes, in the three-dimensional structure of the native protein, a considerable amount of the helical content of this protein. Synthetic analogs of this fragment involving replacement of amino acids with others, and the systematic variation of the relative polarity of side chains along the polypeptide will hopefully give useful information about the relative helix forming and helix breaking capacity of individual amino acids. These effects can be tested by addition of the synthetic analogs to (1-126), and an examination made of the complex formed, if any, for helical structure, activity and, as a fringe benefit, the creation of the antigenic site characteristic of this part of the structure of nuclease. (Parikh, Corley, Sachs, East, Schechter, Anfinsen, Eastlake)

C. Study of the structure and process of reversible denaturation of nuclease

The residue that is most conveniently studied in protein molecules by high resolution nuclear magnetic resonance is histidine. Nuclease contains 4 histidine residues and, in principle, the progress of these can be followed individually during the conversion of the folded nuclease molecule to its denatured, unfolded state by nuclear magnetic resonance measurements as a function of changes in pH, temperature or other denaturing conditions. To refer such changes in individual histidine resonance, it is necessary to relate each of the spectral peaks back to the three-dimensional structure. In order to do this in an unequivocal way, semi-synthetic nuclease derivatives are being prepared in which specific histidine residues are replaced with glycine, thus removing the source of the particular resonance in question. (It has been shown by earlier studies that such histidine-glycine interchanges do not lead to loss in either activity or gross structure.) The semi-synthetic materials which have now been prepared are undergoing thorough purification before the systematic NMR studies of the complexes are undertaken. (East)

In the meantime, it has been shown that during the denaturation of nuclease caused by a change in pH from values above 4.5 to values below 3.2, individual histidine residues can, indeed, be shown to undergo a progressive change from a sterically restricted to an unrestricted environment characteristic of free histidine itself. These studies in conjunction with parallel studies of changes in the intrinsic viscosity, tryptophan fluorescence and optical rotatory properties of nuclease indicate that the structure of this protein, and by inference of others as well, is a highly cooperatively stabilized one and that all of the properties characteristic of the native state appear to "melt out" simultaneously and over a very narrow range of pH change.

Only a single histidine residue and possibly the single tryptophan residue in nuclease show any deviation from the average behavior of all the other structural components that have been systematically investigated. The over-all results make it clear why, over many years, the process of denaturation has been treated as a 2-step process in which one has only native or denatured material and nothing in between. It is simply extremely difficult with available instrumentation to detect the very small and highly transient intermediates that are present at any given point during the explosive denaturation process. (Schechter, Sachs, Epstein, Eastlake)

II. Studies on polymeric protein systems of biological interest

Although most of the work in the laboratory is concerned with the use of model proteins that are monomeric in solution, there is also considerable activity in the investigation of specific aggregation of protein monomers to form biologically active polymers. One example of an enzyme that is active only in the tetrameric form is β-galactosidase. This enzyme is widely distributed throughout the bacterial world and studies have been carried out in the laboratory on the comparative chemical and functional properties of this protein in a variety of organisms. A method was developed for the purification of β-galactosidase from Escherichia coli which is capable of application to extracts of other cells as well. Affinity chromatography, which was largely developed in this laboratory, was applied in this purification. Purification of the multiple forms of the enzyme β-galactosidase by a technique utilizing the very specific binding of the enzyme to the inhibitor p-amino phenylthiogalactoside. This method allows for essentially the one-step purification of this enzyme. A specific resin was prepared which consisted of the analog inhibitor of β-galactosidase p-aminophenylthio-β-D-galactopyranoside which was covalently coupled to sepharose 4B. To obviate steric effects, a hydrocarbon chain was interposed between the inhibitor and the matrix--3,3'-diamino dipropyl amine which had been succinylated. The bound material was eluted with an 0.1 M Sodium borate buffer, pH 10.0.

The method has been applied to the isolation of β-galactosidase of B. megaterium. By hydrodynamic criteria, the subunit molecular weight was 120-130,000 MW. The dimer was 240-250,000 MW. Association was found to be dependent on presence of 5-10% sucrose. The subunit alone was found to be inactive when examined while attached to sepharose. Reactivation upon addition of monomer was achieved. B. megaterium monomer was able to bind E. coli monomers, suggesting some degree of homology between these bacteria. Amino acid composition of B. megaterium was quite similar to that for E. coli. (Steers, Pollard, Cuatrecasas)

Comparison of the enzyme from a number of Enterobacteriaciae was also carried out by studying the immunological cross reactivity of β-galactosidase isolated from them with an antibody prepared against the enzyme from E. coli. It was observed that cross reactivity was general, indicating close similarity between the protein from a variety of species. (Steers, Erickson)

Another protein system that has been under investigation is the group of protein components that form the trichocysts of Paramecium aurelia. A number of mutants of this protozoan are available which exhibit trichocysts on their

outer membranes with varying gross morphology. A method was evolved for the preparation of trichocysts on a large scale. Paramecia were grown in 5 gallon carboys and also in 400 liter batches. Undischarged trichocysts were obtained by suspending the paramecia in solutions of EDTA, disrupting them by homogenization, and then passing the homogenate through Sephadex G-200. Discharged trichocysts were obtained either by the use of dimethylsulfoxide and ethanol or by other methods using EDTA and ATP. Trichocysts were solubilized by various agents. Polyacrylamide electrophoresis and column chromatography were performed on the solubilized proteins. Wild type trichocysts can be solubilized in acid, base, and in denaturing agents such as guanidine hydrochloride, sodium dodecyl sulfate, lithium iodosalicylate. Analysis of the solubilized trichocysts by acrylamide disc electrophoresis in gels containing sodium dodecyl sulfate yielded results comparable to those obtained from trichocysts solubilized by SDS alone, namely, 2 bands with estimated molecular weights of 17,000 and 36,000. Modification procedures such as reduction and alkylation followed by maleylation or maleylation alone will also put this organelle into solution. Employing these procedures, a more detailed analysis of trichocyst proteins is now being undertaken. This work will then be extended to the mutants. (Steers, Pollack)

III. Studies on the regulation of histidine biosynthesis in Salmonella typhimurium

On the basis of our previous work on the kinetics of derepression of the histidine enzymes we postulated that the first enzyme for histidine biosynthesis plays a previously unrecognized role in repression of the histidine system in Salmonella typhimurium. Because it has been known for some time that His-t-RNA is required for repression of the histidine system, it appeared to us that one test of our hypothesis is to determine whether the first enzyme for histidine biosynthesis interacts with His-tRNA. The first enzyme for histidine biosynthesis (G enzyme) was purified on a large scale from extracts of Salmonella typhimurium which had been grown under conditions in which the histidine enzymes are derepressed. His-tRNA was isolated from S. typhimurium by standard procedures. Sephadex columns were set up and tested with known interacting macromolecular systems for use in experiments of the type described by Hummel and Dryer. Another technique for testing the interaction of G enzyme with His-t-RNA, based on the nitrocellulose filter assay of Yarus and Berg, has been developed. Binding experiments done on Sepharose columns and nitrocellulose filters demonstrated that the G enzyme has a high affinity for His-tRNA in vitro (dissociation constant approximately 10^{-9} M). Comparing the binding of ^{3}H-His-tRNA with that of tRNA aminoacylated with other ^{3}H-amino acids disclosed that the binding of the histidyl species of tRNA is favored over that of other species and is dependent upon magnesium-ion concentration. In addition, we found that His-tRNA binds at a site on the enzyme distinct from the catalytic site and the feedback-sensitive site; that the substrates of the enzyme inhibit the binding of His-tRNA, whereas histidine does not do so; that, once a complex has been formed between the enzyme and His-tRNA, the substrates of the enzyme decrease the stability of the complex, whereas histidine is without effect; and that purified enzyme which has a defect in its inhibition by histidine (produced by mutation) displays an altered ability to bind His-tRNA, a finding which may reflect the fact that mutants producing such a defective enzyme display an alteration of the repression process. The finding that

His-tRNA, a macromolecule known to be required for repression of the histidine system, binds with a very high affinity to the first enzyme of the histidine biosynthetic pathway, may be an important clue to understanding the mechanisms which regulate biosynthetic metabolic pathways. (Kovach, Blasi, Goldberger, Barton)

To determine the nature of the genetic basis of the effect of the G-enzyme on repression of the histidine operon, an episome bearing a wild type G gene was introduced into a feedback resistant strain. The merodiploid was tested for its ability to be repressed by TRA. The proper strains were constructed for selection of an episome bearing a G gene with a mutation to feedback resistance. Whereas the feedback resistant strain itself was not repressible by TRA, the episome-bearing strain was repressible by TRA. This finding indicates that the effect of the G enzyme is trans. We, therefore, conclude that the G enzyme exerts its effect on the repression process not by acting locally, at its site of synthesis, but by acting as a freely diffusible gene product, as expected for a repressor. (Kovach, Vogel, Goldberger)

The mechanism of feedback inhibition of the enzyme phosphoribosyltransferase, which catalyzes the first step of the pathway for histidine biosynthesis, was also investigated by studies of the purified protein in solution. Phosphoribosyltransferase was purified from wild type Salmonella typhimurium and from a derivation of this strain in which a mutation in the first gene of the histidine operon leads to the production of a phosphoribosyltransferase which is not sensitive to inhibition by histidine. Using these two enzymes, we carried out studies involving spectrophotometric titration of phenolic hydroxyl groups, solvent perturbation difference spectroscopy, determination of tryptophan and tyrosine content, ultraviolet spectrofluorometry, and circular dichroic spectroscopy. All studies were done on the enzyme preparations in the absence of histidine and in the presence of histidine at various concentrations to assess the effect of histidine on the conformation of the proteins. (1) In the presence of histidine the enzyme displays a positive ultraviolet difference spectrum. Analysis of this effect by the solvent perturbation method indicates a burying of chromophores upon addition of histidine. Spectrophotometric titration of the ionization of tyrosyl hydroxyl groups discloses that histidine prevents ionization of 12 tyrosyl residues in the enzyme. (2) Addition of histidine to the enzyme causes a shift in the fluorescence emission spectrum, the extent of which is dependent upon histidine concentration. Titration of the shift with histidine reveals a non-linear behavior, suggesting a cooperativity in this effect of histidine. (3) Addition of histidine to the enzyme does not significantly alter the circular dichroic spectrum of the enzyme. This indicates that histidine does not cause a significant change in the secondary structure of the enzyme. (4) Histidine-insensitive enzyme, isolated from a feedback-resistant mutant, does not show the change in fluor escence which is produced by histidine in the wild type enzyme. The mutant enzyme does, however, bind histidine, judging from the fact that it, like the wild type enzyme, is protected by histidine against denaturation by urea. We conclude that inhibition of phosphoribosyltransferase by histidine is brought about by a change in the conformation of the enzyme. Although phosphoribosyltransferase from a feedback-resistant mutant is able to bind histidine, it is unable to undergo the conformational change characteristic of the wild type enzyme. Thus, the failure of the mutant enzyme to be inhibited

by histidine is probably due to an alteration of its structure which does not permit a conformational change to occur when it binds histidine.
(Blasi, Goldberger, Aloj)

Serial No. NIAMD-LCB-1
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the relationship between the amino acid sequences and the functional three-dimensional structures of staphylococcal nuclease and bovine pancreatic ribonuclease A: the mechanism of the specific folding of protein.

Previous Serial Number: NIAMD-LCB-1

Principal Investigator: Hiroshi Taniuchi

Other Investigators: Generoso Andria
Janice L. Bohnert

Man Years:

Total: 2.8
Professional: 1.7
Others: 1.1

Project Description:

Objectives: To find the quantitative relationship between the amino acid sequence and the functional three-dimensional structure of protein.

Methods Employed and Major Findings: As reported in previous years, various inactive and disordered fragments of staphylococcal nuclease (149 residues, no sulfhydryl groups nor disulfide bonds) form two types of enzymically active structure resembling nuclease by non-covalent interaction. The first structure contains the discontinuity of the polypeptide chain between residues 48 and 50 and the second one possesses the discontinuity of the polypeptide chain somewhere between residues 113 and 124. When Nuclease-(1-126)(residues 1 to 126) is mixed with Nuclease-T-(49,50-149)(the mixture of two fragments of residues 49 to 149 and 50 to 149), two alternative enzymically active complexes corresponding to the first and second structures are simultaneously formed at approximately equimolar ratio and the redundant portions of the polypeptide chains appeared to protrude flexibly from the ordered structures. On the basis of these observations the working hypothesis is made that almost the entire amino acid sequence of nuclease is essential to determine unique folding and the native conformation of nuclease cannot be formed during biosynthesis until the polypeptide chain has been extended beyond residue 126, that only limited portions of the amino acid sequence of nuclease are permissible for the cleavage without destruction

of the structure and these are between residues 48 and 50 and somewhere between residues 113 and 124 and that a structure similar to nuclease may be formed in several ways on the basis of thermodynamic equilibrium when the requirement of the information of the amino acid sequence to determine the unique functional structure of nuclease is fulfilled.

The present studies were carried out in order to test the formation of an enzymically active structure by the complementation of three fragments of nuclease and in order to establish that the complementation of nuclease fragments is an equilibrium system.

Nuclease-T-(6-48), Nuclease-(49,50-126) and Nuclease-(99-149) were prepared by the method described in the previous report. It was observed that the three fragments bind below 20° to generate very low but clearly intrinsic enzymic activity. The observations indicate that the simultaneous presence of the discontinuity at two permissible points of the polypeptide chain decrease the stability of the structure as compared with the other complexes containing the discontinuity of the polypeptide chain at only one permissible point but the information of the unique folding still somehow remains.

Nuclease-Tb, ((6-48)+(50-149)), was separated from Nuclease-Ta, (6-48)+(49-149)), on a phosphocellulose column by slow gradient elution with ammonium acetate buffer. Nuclease-Tb was acetylated in saturated sodium acetate, pH 5.8, at 0° with acetic anhydride-1-^{14}C. Acetylated Nuclease-T-(50-149) was separated by the method described in previous years. The amino acid analysis of radioactive peptides obtained from the tryptic digests of acetylated Nuclease-T-(50-149) was performed. The results indicated that both α-amino and ϵ-amino groups of residues 50 and 127, respectively, were acetylated together with small amount of ϵ-amino groups of several other residues. Radioactive Nuclease-T was reconstituted by mixing of cold Nuclease-T-(6-48) and radioactive Nuclease-T-(50-149) and purified by phosphocellulose column chromatography after trypsin digestion in the presence of pdTp and Ca^{++} to remove unbound fragments. Further purification of radioactive Nuclease-T' was carried out by affinity column (pdTp-sepharose) chromatography. Radioactive Nuclease-T' and cold Nuclease-T-(50-149) were mixed and incubated at 4°, 10°, 15° and 20° for timed periods. Nuclease-T-(50-149) was separated from Nuclease-T' by affinity column chromatography. The incorporation of radioactive Nuclease-T-(50-149) of Nuclease-T' into Nuclease-T-(50-149) fraction was determined. The results indicated that Nuclease-T-(50-149) incorporated in the structure of Nuclease-T' exchanges with disordered Nuclease-T-(50-149) outside Nuclease-T' and the rate of the exchange increases markedly as temperature increases.

In the last year it was reported that the removal of the four residues from the carboxyl end of reduced bovine pancreatic ribonuclease A causes destruction of the information to form "correct" disulfide bonds upon oxidation. In this year Generoso Andria has mainly worked to extend the studies on the mechanism of correct pairing of disulfide bonds of RNase A. Enzymically

inactive des-(119-124)-RNase was prepared from des-(121-124)-RNase (see the report of the past year) by the method of M.C. Lin (J. Biol. Chem. 245, 6726 (1970). Des-(119-124)-RNase was mixed with the tryptic digest of HCOOOH oxidized RNase. The enzymically active complex composed of des-(119-124)-RNase and RNase-(105-124)(residues 105 to 124) was separated by gel filtration at pH 8. The separation of RNase-(105-124) was performed by the second gel filtration of the complex in 50% acetic acid. Reduced des-(119-124)-RNase (0.2 mg/ml) was air-oxidized in the absence and presence of RNase-(105-124)(pH 7, 25°). In both cases the product showed a featureless circular dichroism at 220 to 300 mμ, different from that of native des-(119-124)-RNase and did not generate significant enzymic activity with RNase-(105-124). The results indicated the formation of randomly paired disulfide bonds in reduced-reoxidized des-(119-124)-RNase. The randomly oxidized des-(119-124)-RNase (0.1 mg/ml) was incubated in the absence and presence of RNase-(105-124) with disulfide interchange enzyme and 0.4 mM β-mercaptoethanol. Only the latter mixture formed a material complementing with RNase-(105-124) (25% yield on the basis of generated enzymic activity). Thus, reduced des-(119-124)-RNase and RNase-(105-124) do not appear to interact in a specific way, to lead to correct pairing of half-cystine residues upon oxidation. The structure of native des-(119-124)-RNase is stabilized by non-covalent binding to RNase-(105-124) and is formed from incorrectly oxidized des-(119-124)-RNase by disulfide interchange in the presence of RNase-(105-124).

Significance to Biomedical Research: The object is to understand the significance of the amino acid sequence of proteins, determined by the genetic information and selected by nature during evolution, in terms of three-dimensional structure and function. The developed system of intrachain complementation of nuclease and ribonuclease A is governed by thermodynamic equilibrium and provides useful testing objective to study quantitatively the relation of the amino acid sequence to the functional three-dimensional structure of protein by chemical and physical means.

Proposed Course of Project:

1) Study on thermodynamic parameters concerning interactions between Nuclease-T-(6-48) and Nuclease-T-(50-149) or Nuclease-T-(49-149) and between Nuclease-(1-126) and Nuclease-(111-149).

2) Study on the complementation of fragments of ribonuclease A in order to understand the mechanism of correct pairing of disulfide bonds.

3) Develop the fragment complementation system of other enzymes with higher molecular weight in order to investigate the general principle underlying the specific folding of globular protein.

4) Examination on amide groups of dicarboxylic amino acid residues of nuclease Foggi to complete the work on the amino acid sequence.

Publications:

Taniuchi, H., Formation of randomly paired disulfide bonds in des-(121-124)-ribonuclease after reduction and reoxidation. J. Biol. Chem. 245: 5459-5468, 1970.

Taniuchi, H., and Anfinsen, C.B.: Simultaneous formation of two alternative enzymically active structures by complementation of two overlapping fragments of staphylococcal nuclease. J. Biol. Chem., in press, 1971.

Cone, J.L., Cusumano, C.L., Taniuchi, H., and Anfinsen, C.B.: Staphylococcal nuclease (Foggi strain) II. The amino acid sequence. J. Biol. Chem., in press, 1971.

Serial No. NIAMD-LCB-2
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: X-ray studies on the three-dimensional structure of Nuclease-T', an enzymically active derivative formed by complementation of two fragments of staphylococcal nuclease.

Previous Serial Number: NIAMD-LCB-7

Principal Investigators: Hiroshi Taniuchi
Christian B. Anfinsen

Man Years:

Total: 0.4
Professional: 0.3
Others: 0.1

Project Description:

Objectives: To investigate the molecular structure of Nuclease-T' by X-ray crystallographic methods.

Methods Employed and Major Findings: Two fragments of staphylococcal nuclease, Nuclease-T-(6-48)(residues 6 to 48) and Nuclease-T-(50-149)(residues 50 to 149) were prepared by limited digestion of nuclease with trypsin in the presence of thymidine-3',5'-diphosphate (pdTp) and Ca^{++} and purified as described in previous reports. Nuclease-T' was reconstituted by mixing of Nuclease-T-(6-48) and Nuclease-(50-149) at approximately equimolar ratio. Unbound, excess fragments were removed by digestion with trypsin in the presence of pdTp and Ca^{++}. Nuclease-T' ((6-48)+(50-149)) was purified by phosphocellulose column chromatography and was crystallized in 0.0105 M potassium phosphate buffer, pH 8.3 with 38% 2-methyl-2,4-pentanediol in the presence of pdTp and Ca^{++}, at 4°. The crystals have the form of fine needles and are very unstable at 25°.

Significance to Biomedical Research: The availability of a Nuclease-T' crystal suitable for X-ray crystallographic studies would permit detailed studies on structure-function relations. Since chemically synthesized fragments containing some replaced residues (see 1969-70 Serial No. NIAMD-LCB-2) could be used as one or both of the components of crystalline Nuclease-T', the role of individual residues to structure-function relations of nuclease could be evaluated at the atomic level.

Proposed Course of Project: Search for conditions to make Nuclease-T' crystals suitable for X-ray work. X-ray crystallographic studies would be performed with cooperation of Dr. David R. Davies (NIH, NIAMD, LMB). The crystallization of another enzymically active complex, ((1-123)+(114-149)), of staphylococcal nuclease (1969-70 Serial No. NIAMD-LCB-1) also will be tried.

Serial No. NIAMD-LCB-3
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Structure-Function Studies on Staphylococcal Nuclease and Nuclease-T.

Previous Serial Number: NIAMD-LCB-2

Principal Investigator: Irwin M. Chaiken

Other Investigators: Guillermo Sanchez
Christian B. Anfinsen

Man Years:

Total: 0.6
Professional: 0.6

Project Description:

Objectives:
1) To investigate structure-function properties of staphylococcal nuclease-T, the noncovalent complex of the two nuclease-derived peptide fragments containing, respectively, residues 6 through 48 and 49 through 149. Specifically, studies were carried out on the effects on nuclease-T activity of both 1) chemical modification of native fragment 6-48 and 2) addition of the ligands, Ca^{++} and deoxythymidine-3',5'-diphosphate.
2) To correlate information for native nuclease-T with that obtained by solid phase synthesis and characterization of analogue forms of the fragment corresponding to residues 6 through 47 of nuclease.
3) To investigate the possible solid phase or conventional synthesis of other fragments useful in the study of nuclease and nuclease-T.

Methods Employed: The native fragments, nuclease-(6-48) and -(49-149), were obtained from native staphylococcal nuclease by the established procedures of selective trypsin digestion, phosphocellulose chromatography, Bio-Gel fractionation and further phosphocellulose chromatography.
Preparation of synthetic-(6-47) and analogues was accomplished using the solid phase synthetic procedure of Merrifield. Adaptations of the normally used procedure have been employed occasionally.
Studies with both native and synthetic fragments have necessitated enzymic activity assays, fluorescence measurements, and immunochemical characterization.

Major Findings: Study of native nuclease-T' has shown a small but repeatable enhancement of enzymic activity by the two stabilizing ligands, Ca^{++} and pdTp. A corresponding effect has been shown for the mixture of synthetic-(6-47) and nuclease-(49-149); and a more dramatic effect occurs with the mixture of nuclease-(49-149) with some chemically modified nuclease-(6-48) forms and several analogues of synthetic-(6-47), especially with analogues which generate low-activity complexes with the native fragment in the absence of ligands. As a result of such studies, the structure-stabilizing involvement of several nuclease residues, including glutamic acid residue 10, proline 11, alanine 12, isoleucine 18, and tyrosine 27 has been described. All of these results can be rationalized with the atomic model of staphylococcal nuclease obtained by X-ray crystallographic studies at M.I.T.

Significance to Biomedical Research: These investigations have formed a hopeful beginning of an overall attempt to develop general rules for the role of all amino acids in the structure and activity of nuclease and, perhaps, other proteins as well.

Proposed Course of Project:

1) Systematic studies of properties of the semisynthetic mixtures of nuclease-(49-149) with the various, already-prepared synthetic-(6-47) analogues, especially regarding comparative enzymic activity on low and high molecular weight substrates, temperature stability of the semisynthetic complexes, when formed, and the effect of ligands on both of these properties.

2) Extension of available structure-function information, and generation of new areas of study, by preparation of further analogues of synthetic-(6-47).

3) Synthesis of parts of the 6-47 region by solid phase and conventional methods as an alternative approach to the preparation of synthetic analogues of the (6-47) region. This work is in progress in collaboration with Dr. Carlo Di Bello of LCB, NIAMD.

4) Efforts to synthesize the peptides corresponding to whole nuclease and nuclease-(49-149) as part of an overall effort to expand the utilization of synthesis to engineer protein synthetic modification approaches for structure-function studies.

5) Correlation, insofar as possible, with other protein chemical and enzymic studies on native nuclease, especially the isolation of a covalent catalytic intermediate (in collaboration with Dr. Carlo Di Bello of LCB, NIAMD) and chemical modification studies.

6) Studies, in progress in collaboration with Drs. David East and David Sachs of LCB, NIAMD, to find means for facilitation of solid phase synthetic studies by development of synthetic method modifications.

Publications:

Chaiken, I.M., and Anfinsen, C.B., A Solid Phase Synthetic Study of Structure-Function Relationships in the Amino-Terminal Region of Staphylococcal Nuclease, J. Biol. Chem. 245, 4718 (1970).

Chaiken, I.M., and Anfinsen, C.B., A Solid Phase Synthetic Study of the Active Site Region of Staphylococcal Nuclease-T', J. Biol. Chem. 246 (April 10, 1971), in press.

Serial No. NIAMD-LCB-4
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Preparation and studies of semisynthetic noncovalent protein complexes.

Previous Serial Number: NIAMD-LCB-2

Principal Investigator: Irwin M. Chaiken

Other Investigators: Jack S. Cohen

Cooperating Units: 1) Physical Sciences Laboratory, Division of Computer Research and Technology, NIH (Jack S. Cohen: some of this work reported in annual report PSL-DCRT-5.6).
2) Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada (Murray H. Freedman).

Man Years:

Total: 0.5
Professional: 0.5

Project Description:

Objectives:
1) To prepare peptides, by solid phase synthesis, which correspond to native protein fragments that participate in noncovalent complexes with complementing protein fragments.
2) To use the functional property of fragment complementation to prepare purified semisynthetic protein complexes using these solid phase synthetic fragments.
3) To characterize the semisynthetic complexes for normal as well as for analogue protein sequences.
4) To describe the use of semisynthetic protein complexes for the application of peptide synthesis to the preparation of materials useful for careful studies of analogue protein forms.

Methods Employed: The synthetic-(6-47) peptide corresponding to residues 6 through 47 of staphylococcal nuclease has been combined with the native fragment, nuclease-(49-149), and subsequently isolated as part of a resultant highly active enzyme complex after selective trypsin digestion of most non-complementing fragments. The purified complex has been characterized for enzymic and structural features and compared in these with the native complex of nuclease-(6-48) and nuclease-(49-149). The preparation of the semisynthe-

tic complex of normal sequence has been used as a model for the preparation of analogue complexes, as for example that containing the synthetic analogue, [Asp^{43}]-synthetic-(6-47). The latter peptide binds to nuclease-(49-149) but generates no enzymic activity.

The synthetic peptide corresponding to residues 1 through 15 of bovine pancreatic ribonuclease has been prepared by the solid phase method and used to complement with native ribonuclease S-protein (residues 21-124) to yield semisynthetic ribonuclease S'. The latter is isolated from the semisynthetic mixture by Sulfoethyl-Sephadex fractionation. Synthetic ribonuclease-(1-15) prepared with ^{13}C enriched phenylalanine to obtain the peptide with phenylalanine 8 with 13% ^{13}C has been used to study ribonuclease S by ^{13}C-nuclear magnetic resonance (these studies are being carried out in collaboration with Drs. Jack S. Cohen and Murray H. Freedman).

Major Findings: Semisynthetic Nuclease-T', the complex of synthetic-(6-47) and nuclease-(49-149), was obtained with 90% specific activity and properties, including catalytic specificity, Km, fluorescence characteristics, and temperature stability, closely similar to those of the native nuclease-T' complex. Preliminary efforts indicate the possibility of obtaining analogue complexes. For [Asp^{43}]-synthetic-(6-47), a possible complex would allow the careful study of a purified species closely similar to the normal semisynthetic complex but containing a crucial replacement of the normally occurring glutamic acid 43. This latter residue is indicated by the crystallographic model of nuclease to be in the active site region and by previous synthetic studies to be essential for enzymic activity.

Semisynthetic ribonuclease-S', containing ^{13}C-enriched phenylalanine 8, was obtained in a form about equally as active as native ribonuclease S'. The semisynthetic complex has a ^{13}C-nuclear magnetic resonance spectrum which, taken with information of planned experiments, hopefully will allow not only specific conclusions about the disposition of phenylalanine 8 in ribonuclease S but also an indication of what kinds of information can be obtained with further studies of ^{13}C-NMR of peptides and proteins.

Significance to Biomedical Research: The ability to prepare highly active semisynthetic protein complexes should provide a means for adapting solid phase peptide synthesis of long polypeptide fragments to ensure study of fairly pure protein derivatives. Conclusions involving analogue complexes should be possible with increased confidence and clarity over what obtains when dealing with crude solid phase synthetic peptides.

Proposed Course of Project:

1) Isolation and characterization of selected analogue semisynthetic nuclease-T' species, for analogues of synthetic-(6-47) demonstrating important properties.

2) Continuance of ^{13}C-NMR studies on [^{13}C-Phe_8]-semisynthetic ribonuclease S' and preparation and characterization of other ^{13}C-enriched S' species.

3) Preparation of other kinds of semisynthetic complexes.

Publications:

Chaiken, I.M., Purification and properties of solid phase semisynthetic staphylococcal nuclease-T', J. Biol. Chem. 246 (May, 1971), in press.

Chaiken, I.M., Preparation and studies of semisynthetic noncovalent enzyme complexes, Fed. Proc. (1971), to be presented in June, 1971 at the San Francisco meeting of the ASBC.

Freedman, M.H., Cohen, J.S., and Chaiken, I.M., Carbon-13 Fourier transform nuclear magnetic resonance studies of proteins, Biochem. Biophys. Res. Commun. 42 (6), 1148 (1971).

Serial No. NIAMD-LCB-5
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Spectroscopic studies of the folding of staphylococcal nuclease.

Previous Serial Number: NIAMD-LCB-8 and 9

Principal Investigators: Henry F. Epstein
Alan N. Schechter
Christian B. Anfinsen
Ann Eastlake

Cooperating Units: Raymond F. Chen (H:LTD)
Jack S. Cohen (DCRT:PSL)

Man Years:

Total: 1.6
Professional: 1.6

Project Description:

Objectives: Knowledge of the mechanism by which the primary sequence of amino acids leads to the formation of the biologically active, three-dimensional structure of native staphylococcal nuclease is the principal goal of these studies. Understanding of the pathways of enzyme folding can come only from a large number of experimental approaches. The immediate aim of this project is to utilize spectroscopic techniques which focus upon local regions of the protein molecule and can, thus, provide detailed information on how individual amino acid residues behave during folding. Future studies are envisaged which will apply these spectroscopic techniques to semi-synthetic analogues of staphylococcal nuclease in which deletions or other modifications of the primary sequence have been effected. In this manner, the relationship between the genetically determined amino acid sequence and the resultant native structure may be clarified.

Methods Employed: Fluorometric titrations and energy transfer studies are performed in an Aminco-Bowman spectrophotofluorometer. Kinetic measurements were obtained with a stopped-flow modification of an Aminco-Bowman spectrophotofluorimeter. Proton magnetic resonance studies were performed on a Varian Associates HR-220 spectrometer. The Modelaide facility of the Division of Computer Research and Technology has been extensively used in the analysis of the magnetic resonance spectra.

Classical protein chemistry techniques such as amino acid analyses and

peptide mapping have been used to determine the structure of modified proteins. Organic chemical methods have been used to synthesize nucleoside analogue phosphates which can be competitive inhibitiors of the enzyme.

Major Findings: The folding of acidified staphylococcal nuclease, upon neutralization, was studied in a stopped-flow spectrofluorometer by measuring the increase in tryptophanyl fluorescence during renaturation. At 25°C and 0.1 ionic strength the fluorescence change, under conditions in which folding alone occurs, may be described by two first-order rate processes with half-times of 55 and 350 msec. No significant change in either rate was effected in varying the initial pH from 3.2 to 3.8 (corresponding to 0 to 80% levels of folding) or the initial ionic strength from 0.001 to 0.1. In contrast, the overall rate of folding is dependent upon temperature. The half-time of the slower rate process decreases from 600 msec at 13°C to 150 msec at 38°C; the half-time of the faster rate process does not change significantly over this temperature range. Thus the faster process has $\Delta S^{\ddagger}$=-58 e.u., while the slower process has $\Delta H^{\ddagger}$=7.9 Kcal/mole and $\Delta S^{\ddagger}$=-33 e.u. These activation parameters suggest that the two processes may correspond to a sequence involving nucleation of ordered structure followed by the formation of hydrophic interactions. In particular, the tryptophanyl indole ring lies in a hydrophobic pocket between two alpha-helices.

The reversible unfolding and folding equilibrium of staphylococcal nuclease in the acid transition has been studied using 220 MHz proton magnetic resonance spectroscopy. The imidazole Cz protons of the four histidine residues may be observed as individual resonances in this transition. The change in areas of three histidine resonances as a function of pH follow the same equilibrium as the change of fluorescence of the single tryptophan residue or of reduced viscosity. This equilibrium with a half-change at about pH 3.9 probably represents the major structural rearrangement during nuclease folding. In addition, a fourth histidine resonance loses about one-half of its area between pH 4 and 5. This transition which correlates with the asymmetry of both the tryptophan and tyrosine fluorescence emissions above pH 4, may represent a local pertubation of structure. These findings furnish equilibrium evidence that the folding of this enzyme from the unfolded state to the native structure may involve intermediate forms.

Equilibrium studies involving viscosimetry and circular dichroism of the peptide bonds as a function of pH are being pursued in order to determine how the overall structure of the enzyme changes during the acid transition. The reduced viscosity as a function of pH shows no change above pH 4.0 and sharply increases with a pH of half-change at 3.9.

Another experimental approach involves the synthesis of nucleoside analogue phosphates, in particular deoxythiothymidine-3'5'-diphosphate. This analogue of TDP has an absorption maximum at 340 nm, far away from the protein absorption maximum. The binding of TDP and Ca^{+2} do not affect the irreversible folding of neutralized protein. Thus, the binding of thio TDP and Ca^{+2} can serve as a monitor of the formation of the active site of staphylococcal nuclease during folding.

A further approach is the use of fluorescent labels placed on specific

amino acids. The selective nitration of tyrosines 115 and 85 has been previously accomplished in this laboratory. The nitro groups may be reduced to amino groups. These amino groups have a pK of 4.75 in contrast to the much higher pK's of the α-amino and ϵ-amino groups. Thus, SN2 reagents can selectively react with these amino groups between pH 4.5 and 5. N-methyl anthraniloyl chloride has been used to place a highly fluorescent group (quantum yield of ~ 0.5 and emission-maximum at 420 nm) on these aminotyrosine residues. The anthranilate group is an excellent acceptor of electronic energy from the single tryptophan. Equilibrium studies by energy transfer between tryptophan and randomly placed anthilate groups has been performed for urea denaturation.

Significance to Biomedical Research: The precise relationship between the genetically determined primary sequence and the active structure of a protein remains one of the major unsolved links in the understanding of protein biosynthesis. Such knowledge may ultimately provide us with the ability to engineer new proteins as well as to clarify how mutant proteins cause human disease. These studies of the dynamic aspects of protein folding and structure will contribute to the knowledge of these relationships.

Proposed Course of Project: The energy transfer and nucleotide analogue binding studies are being continued in order to understand how different parts of staphylococcal nuclease relate to one another during the folding process. Future experiments relating the change in amino acid sequence to changes in the folding mechanism by use of these spectroscopic methods is envisaged.

Serial No. NIAMD-LCB-6
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Synthetic analogues of a helical portion of staphylococcal nuclease: enzymologic and immunologic properties.

Previous Serial Number: NONE

Principal Investigators: David H. Sachs
Alan N. Schechter
Christian B. Anfinsen
Ann Eastlake

Man Years:

Total: 1.6
Professional: 1.1
Others: 0.5

Project Description:

Objectives: The C-terminal cyanogen bromide fragment of staphylococcal nuclease (Piece E), a polypeptide of 50 amino acids, binds to another enzymatically inactive fragment of staphylococcal nuclease (Piece 1-126) to generate activity. At least one of the antigenic sites of intact nuclease has been localized to this Piece E fragment. In addition, the crystallographic model of nuclease shows an α-helical region of 14 amino acids (122-135) within this C-terminal sequence. We wish to study synthetic analogs of Piece E with amino acid substitutions at given points in the helical region in order to determine the importance, if any, of this helical conformation for generation of both biological properties of this fragment--binding to Piece 1-126 to generate enzymatic activity and formation of an antigenic site.

Methods Employed: Piece 1-126 and Piece E have been prepared from pure staphylococcal nuclease by procedures developed previously in this laboratory. Antisera have been raised in rabbits and goats using native staphylococcal nuclease and Pieces 1-126 and E as antigens. The reactivities of these sera have been characterized by immunodiffusion analyses, precipitin curves, and passive hemaglutination assays.

Solid phase synthesis of the sequence corresponding to amino acids 111 to 145 of native nuclease has been carried out following the method of Merrifield. Separate syntheses with amino acid substitutions at given positions in the "helical region" of this polypeptide have also been carried out simultaneously. The substitutions were made at positions which appear to be "outside" in the crystallographic model of nuclease and which might therefore be

expected to affect antigenicity of the fragments without affecting binding. Multiple alanine substitutions were made in one analogue in order to favor helix formation.

Our work with solid phase synthesis has also led to investigations of several basic problems in the solid phase synthetic scheme. These investigations include modification of the method of washing the resin between steps, and determination of the optimal conditions for deblocking.

Major results:

Antibody preparation: Precipitating antiserum against native Piece E has been produced. In addition, a population of antibodies specific for the Piece E region have been detected by passive hemagglutination analysis in antisera against native nuclease. No such population could be detected in the antiserum against Piece 1-126, indicating that there is at least one antigenic site in native nuclease beyond residue 126.

Synthetic analogs: The synthetic polypeptide corresponding to the native sequence has been cleaved from the solid phase resin and partially purified by gel filtration. On addition ot Piece 1-126 a small but definite increase in activity was detected. However, addition of this partially purified synthetic Piece E to native Piece E resulted in a decrease in the maximal activity generated by the latter on addition to Piece 1-126. This indicates that other inhibitory polypeptides are present in the synthetic product--undoubtedly polypeptides of slightly different structure which bind to Piece 1-126 but are not active.

Methodologic studies: The use of a methanol wash between methylene chloride washes, to physically "shrink" the resin, has been found to improve markedly the washout of contaminants. Studies of deblocking conditions have shown that much shorter times of acid treatment produce adequate release of free amino groups with much less loss of peptide from the resin.

Significance to Biomedical Research: Chemical synthesis of polypeptides and proteins, in addition to its practical uses, should provide a means to answer basic questions about the physicochemical basis of the biologic properties of proteins. This study, in particular, is aimed at determining the importance of helical conformation for generation of enzymatic activity and for production of antigenicity.

Proposed Course: Further purification of the synthetic native sequence by physical and chemical methods will be monitored by following the enzymatic activity and the disappearance of inhibitory activity as well as the presence of antigenic activity. The same scheme will then be applied to each of the synthetic analogs and their properties compared and related to conformational and structural differences.

Serial No. NIAMD-LCB-7
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Nuclear magnetic resonance studies of staphylococcal nuclease, bovine pancreatic ribonuclease, and imidazole compounds.

Previous Serial Number: NIAMD-LCB-10

Principal Investigator: Alan N. Schechter
David H. Sachs
Ann Eastlake

Cooperating Unit: LPS, DCRT (Dr. Cohen, Mr. Shrager, and Mrs. McNeil)

Man Years:

Total: 1.0
Professional: 0.6
Other: 0.4

Project Description:

Objectives: Recent work has shown that nuclear magnetic resonance studies give information useful to understanding the structure of proteins in solution and the interactions of proteins with substrates and other ligands. At high sensitivity it is possible to resolve signals from the different aromatic amino acid residues and make inferences about the electronic environment of these residues in the three-dimensional structure of the protein. These inferences may be tested by studies of suitable chemical model compounds and by computation of the data with various mathematical models. Further, the present availability of even more sensitive instruments, data averaging methods, and high speed data processing will expand the usefulness of this approach to the study of macromolecular structure.

Methods Employed: The Varian 220 MHz nuclear magnetic spectrometer with a probe for recording proton resonances has been used for most of our work. Protein samples have been exchanged with pure D_2O and residual protons have been measured and recorded on a Varian time averaging computer. pH titrations have been accomplished by measuring time-averaged spectra at closely spaced pH values. Temperature, ionic strength, and the concentration of substrate analogs have been used as independent variables. Selected chemical modifications of proteins have also been accomplished for the purpose of identifying certain resonances.

Many spectra have been digitized, recorded on magnetic tape, and then

analyzed into their component Lorentzian curves with an IBM 360/50 computer and 22 50 display so that accurate values of chemical shift, area, and peak width could be obtained. Theoretical titration curves of chemical shift vs. pH for the individual resonances have been computed for the Henderson-Hasselbalch relationship and for interacting titrating groups. These curves have been compared to our data by computer fitting.

L-Histidine and various imidazole derivatives have been obtained or synthesized and the proton resonances of these compounds have been studied on the A-60 spectrometer.

Major Findings: The imidazole C2 protons of the four histidine residues of staphylococcal nuclease have been resolved from other resonances and the microscopic pK values of these residues have been established.

The imidazole protons of bovine pancreatic ribonuclease have been studied in an analogous way. However, only one of the four titration curves fits the Henderson-Hasselbalch relationship so that a simple pK could be derived. Two of the curves demonstrate inflections between pH 4 and 5. These inflections indicate that these histidine residues interact electronically with neighboring charged groups. Computer fittings for various interacting models suggest that the best model to describe the results is one in which each of the two histidine residues is interacting with an aspartic or glutamic acid residue. Furthermore, there is evidence that one of the two may also be interacting with a lysine residue. The fourth histidine residue is even more complicated in its interactions and a suitable model has not yet been derived. The effects of substrate analogs, phosphate ions, sulfate ions and other interacting groups have also been measured. Ribonuclease-S has also been prepared and studied.

The titration curves for the C2 and C4 protons of several imidazole derivatives have been studied. The effects of the protonation of neighboring charged groups on the titration of the ring protons has been observed and accounted for by the computer models noted above. These results support and extend the interpretation of the data for ribonuclease.

Significance to Biomedical Research: These new methods allow the study of the behavior of different parts of a protein molecule in solution--thus combining advantages of the usual spectroscopic methods and X-ray crystallography. The results from the imidazole compounds and ribonuclease indicate that the imidazole proton titration curves are sensitive probes to electronic interactions within a small compound or even a protein. The work with substrate analogs is allowing a definition of electronic aspects of their interactions with different parts of the protein molecule. These methods will improve our understanding of the mechanism of action of these, and possibly other, proteins.

Proposed Course of Project: The analysis by computer-fitting of the titration curves in the presence of substrate analog and other ligands will be continued. Further work will be done to identify each of the C2 resonances in each protein. These methods may be extended to other proteins.

Publications:

Cohen, J.S., Shrager, R.I., McNeel, M., and Schechter, A.N.: On-line computer-assisted analysis of 220 MHz NMR data of protein imidazole resonances. Biochem. Biophys. Res. Comm. 40, 144-151, 1970.

Cohen, J.S., Shrager, R.I., McNeel, M. and Schechter, A.N.: Proton magnetic resonance studies at 220 MHz of the histidine residues of staphylococcal nuclease. Nature 228, 642-644, 1970.

Serial No. NIAMD-LCB-8
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Solid phase synthetic studies on staphylococcal nuclease.

Previous Serial Number: NONE

Principal Investigator: David East

Other Investigator: Christian B. Anfinsen

Man Years:

Total: 1.1
Professional: 1.1

Project Description:

Objectives:

1) To assign uniquely the histidine C-2 proton NMR peaks of staphylococcal nuclease.

2) To determine whether or not the C-terminal helix of nuclease is necessary for enzyme activity.

3) To investigate the use of energy transfer between chromophores as a probe for following conformational changes in nuclease.

Methods, Major Findings, and Proposed Course:

1) Of the four histidine C-2 proton NMR peaks of nuclease only that of His 124 is assigned. The peaks of the active complex of fragments 6-47 and 49-149 almost coincide with those of nuclease (Jardetsky, unpublished). Fragment 49-149 also gives active complexes with the 6-47 analogue 6-47 Gly 46 His 8, and with 9-47. The peptides following were made by the solid phase method (Merrifield: JACS 85, 2149):

6-47 His 46, Gly 8
6-47 Gly 46, His 8
6-47 Gly 46, Gly 8
6-47 His 46, His 8 (the native sequence).

They will be complemented with 49-149 and the NMR spectra will be obtained. These will uniquely assign all four Histidine NMR peaks.

2) The nuclease crystal structure includes three helices, the C-terminal helix involving residues 121-134. The nuclease fragment 99-149 (also shortened synthetic analogues e.g. 148-116 Phe 140) forms an active complex with 1-126. We believe these complexes in solution to have the conformation of crystalline nuclease and the C-terminal helix to be donated by 99-149.

Proline, an imino acid, is not a donor in hydrogen bond formation and thus acts as a 'helix breaker.'

To test the relevance of helix 121-134 to nuclease activity we are synthesizing the following peptides:

148-108 (Phe 140 Pro 132)
148-108 (Phe 140 Pro 130)
148-108 (Phe 140, Pro 132, Pro 130)

Their ability to form active complexes and their helical content when complemented will be measured.

3) The equation for energy transfer between suitable chromophores such as Indole and anthranilate and distance R apart is (Förster: Ann. Physik 2, 55)

$$\text{Transfer} = (1/R)^6 \cdot ([1/R]^6 + [1/R_o]^6)^{-1}$$

(where R_o is about 20 Angstroms) and has been confirmed by experiment (Stryer: Science 162, 526). The residues 140 and 126 and 121 and 134 are only 20 A apart in the native enzyme (F.A. Cotton and workers at M.I.T.). Denaturation of the C-terminal helix (residues 121 to 134) should increase this distance and energy transfer between the above residues would become negligible.

We have begun the solid phase synthesis of peptides

145-108 Trp 140 Lys ϵ-anthranilate 126
145-108 Phe 140 Pys ϵ-anthranilate 134
Trp (for His) 121.

The carbodiimide reaction will couple lysine to anthranilic acid and its emission spectrum will monitor the formation of helix 121-134 when the above peptides are complemented with fragment 1-126.

Significance to Biomedical Research: Since His 46 is close to the active site its NMR spectrum and titration curve may be used as a probe for the catalytic site of nuclease.

The synthesis of non-helical fragments 148-108 will enable us to correlate function with a major structural component of many proteins, the alpha helix. The energy transfer experiments if successful will allow one to directly follow the formation of a helix in a protein, and to obtain the thermodynamic quantities involved. Thus, insight into the protein conformation problem will be gained.

Serial No. NIAMD-LCB-9
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Solid phase synthetic studies on staphylococcal nuclease, its CNBr and tryptic polypeptide fragments.

Previous Serial Number: NIAMD-LCB-3

Principal Investigators: Indu Parikh
Lila Corley

Other Investigators: Christian B. Anfinsen

Man Years:

Total: 1.6
Professional: .6
Other: 1

Project Description:

Objectives:
1) To seek stable tryptophan analogues which could survive the acid treatments necessary in solid phase synthesis.
2) Selective chemical modifications of tryptophan in native nuclease to study the importance of tryptophan in nuclease.
3) To synthesize a) the carboxyl terminal CNBr fragment of nuclease (residues 99 through 149) with tryptophan or its analogue; b) the tryptic fragment P_3 (residues 49 through 149); and c) total synthesis of staphylococcal nuclease.
4) To examine selected analogues of the C-terminal CNBr fragment in their ability to bind to P_{126} (residues 1 through 126) to form an active complex.
5) To seek the antigenic determinants in the CNBr fragment of nuclease.

Methods Employed: Tryptophan auxotroph of Staphylococcus aureus (Foggi strain) was made by standard technique using N-nitrosoguanidine. Production of nuclease by growing mutants in media containing specific tryptophan analogue, namely 7-aza-tryptophan, indicated that tryptophan could be replaced by an appropriate analogue in native nuclease.
Solid phase synthetic method developed by Merrifield and employed in this laboratory for a synthesis of a polypeptide fragment (nuclease residues 6 through 47) by David Ontjes and Christian B. Anfinsen is used in the present studies. The X-ray crystallographic studies on nuclease by F.A. Cotton and co-workers at M.I.T. has been used as a valuable guide line.

Major Findings: A number of analogs of the C-terminal CNBr fragment of nuclease (residues 148-99) have been synthesized and examined for their ability to form a complex with a large fragment of this protein containing residues 1-126. It was known previously that the C-terminal fragments isolated from the natural source could complement residues 1-126 to yield an enzymically active complex. The synthetic analog peptides did not yield activity in crude form but after ion exchange chromatography were able to complement essentially as effectively as the native fragment. It was shown that residues 99-114 and 145-148 could be omitted without significant loss in biological function. The tryptophan residue at position 140 could be replaced with phenylalanine and both activity and antigenicity were retained.

Significance to Biomedical Research: These studies constitute a base line for future work on the synthetic preparation of analogs of a protein which will permit direct examination of the molecular basis of enzymic activity and immunochemical properties.

Proposed Course of Project: This project as such is now terminated but the ideas emerging from it will continue to be applied in related projects (NIAMD-LCB 6 and 8).

Serial No. NIAMD-LCB-10
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on an antigen cross-reacting with Escherichia coli β-galactosidase.

Previous Serial Number: NIAMD-LCB-14

Principal Investigator: Edward Steers Jr.

Cooperating Unit: Robert P. Erickson, Department of Pediatrics, University of California Medical Center, San Francisco, California

Man Years:

Total: .5
Professional: .3
Other: .2

Project Description:

Objectives: To isolate and characterize the antigenic substance which cross-reacts immunologically with the enzyme β-galactosidase from Escherichia coli.

Major Findings: We find that crude extracts of a number of species of Enterobacteriaciae contain an antigen cross-reacting with all anti-β-galactosidase sera available. The antigen separates from β-galactosidase on disc gel electrophoresis and antisera prepared against the polyacrylamide disc gel slices containing β-galactosidase react with the antigen. The antigen was purified by a combination of molecular sieve chromatography and by a column containing antibody attached to an insoluble support. Antisera prepared against the purified antigen cross-reacted with purified preparations of β-galactosidase. The antigen is a nucleoprotein with an amino acid composition and molecular weight distinct from those of β-galactosidase.

Significance to Biomedical Research: This work represents an attempt to characterize a functionally unrelated molecule to an enzyme of metabolic importance. The immunological relationship between these two presumably unrelated substances may help to serve as a model for other systems in which immunological relatedness may serve to identify and aid in the study of antigenic substances of interest.

Proposed Course of Project: Terminated.

Serial No. NIAMD-LCB-11
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Use of affinity chromatography in the purification and characterization of β-galactosidase from Escherichia coli.

Previous Serial Number: NIAMD-LCB-15

Principal Investigator: Edward Steers, Jr.

Other Investigator: Harvey Pollard

Cooperating Unit: Pedro Cuatrecasas, Johns Hopkins School of Medicine

Man Years:

Total: .6
Professional: .4
Other: .2

Project Description:

Objectives: Purification of the multiple forms of the enzyme β-galactosidase by a technique utilizing the very specific binding of the enzyme to the inhibitor para-amino phenyl thio galactoside. This method allows for essentially the one-step purification of this enzyme.

Methods Employed: A specific resin was prepared which consisted of the analog inhibitor of β-galactosidase p-aminophenyl thio-β-D-galactopyranoside which was covalently coupled to sepharose 4B. To obviate steric effects, a hydrocarbon chain was interposed between the inhibitor and the matrix--3,3' diamino dipropyl amine which had been succinylated. The bound material was eluted with an 0.1 M sodium borate buffer, pH 10.0.

Major Findings: Crude extracts of E. coli K12 strain 3300 were passed through a column of sepharose 4B (1.5 cm x 30 cm) to which the substrate analog p-amino phenylthio-β-D-galactopyranoside had been covalently attached. The column bound 100% of the enzyme activity while passing over 90% of the total material through in the void volume. Continued washing with buffer did not elute enzyme. The band enzyme was not eluted with the usual substrate solutions lactose, or o-nitrophenyl β-D-galactopyranoside even when run as saturated solutions. Upon changing the buffer to 0.1 M borate, pH 10.05, the enzyme eluted as a sharp boundary of activity at the interface of the two buffer systems. The β-galactosidase purified by this procedure is fully

active and possesses all of the physical and chemical characteristics of enzyme purified by previous procedures.

The procedure can be carried out on a preparative scale, and the adsorbent may be used several times without appreciable impairment of the binding capacity. The enzyme remains active while bound to the column and it can be readsorbed and eluted without apparent deleterious effects.

Of additional interest is the demonstration that the monomer form of the enzyme previously identified in other preparations of β-galactosidase binds to the same extent as the tetramer form and is co-eluted with the tetramer form at pH 10.

It is apparent from this observation that while the monomer form of β-galactosidase is devoid of any hydrolytic activity it is capable of binding to the substrate analog.

Significance to Biomedical Research: This work represents a continuation of our objectives to characterize the structural features of an enzyme whose regulatory mechanisms have led to significant advances in our concepts of developmental processes.

Proposed Course of Project: Terminated.

Publications:

Steers, E., Jr., Cuatrecasas, P., and Pollard, H., The purification of β-galactosidase from Escherichia coli by affinity chromatography. J. Biol. Chem. 246, 196 (1971).

Serial No. NIAMD-LCB-12
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Purification and Properties of B. megaterium, KM β-galactosidase.

Previous Serial Number: NIAMD-LCB-16

Principal Investigator: Harvey Pollard

Other Investigator: Edward Steers, Jr.

Man Years:

Total: 0.3
Professional: 0.3

Project Description:

Objectives:
1) To study the physical, chemical and biologic properties of β-galactosidase from Bacillus megaterium, KM.
2) To investigate influence of subunit association on reactivity of the enzyme.

Methods:
1) β-galactosidase from lysozyme-EDTA-treated cells was purified to homogeneity by affinity chromatography on a medium consisting of [Sepharose-4B-NH-C_3H_6-NH-C_3H_6-NH-CO-CH_2-CH_2-CO-pNH_2-phenylthio β-D galactoside].
2) Enzyme was eluted from the affinity column with pH 10.5 borate buffer.
3) Enzyme was prepared in dimeric form on sucrose gradients. Molecular weights of monomer and dimer were determined by hydrodynamic methods.
4) Enzyme was attached to sepharose in order to prepare sepharose-bound monomers.
5) Amino acid composition was determined.

Major Findings:
1) By hydrodynamic criteria, the subunit molecular weight was 120-130,000 MW. The dimer was 240-250,000 MW. Association was found to be dependent on presence of 5-10% sucrose.
2) The subunit alone was found to be inactive when examined while attached to sepharose. Reactivation upon addition of monomer was achieved.
3) B. megaterium monomer was able to bind E. coli monomers, suggesting some degree of homology between these bacteria.

4) Amino acid composition of B. megaterium was quite similar to that for E. coli.

Significance to Biomedical Research:

1) This system is homologous with the E. coli system and provides a simpler approach to the problem of subunit-subunit interaction.

2) The clear dependence of activity upon association to the dimer may provide insight into the significance of multisubunit enzymes.

Proposed Course of Project:

1) We hope to find conditions where the dimer is stabilized in absence of monomer so that the monomer-dimer system can be studied by hydrodynamic means.

2) Fluorescent inhibitors may be employed to measure binding as a function of association.

Serial No. NIAMD-LCB-13
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Isolation and characterization of trichocysts from Paramecium aurelia.

Previous Serial Number: NIAMD-LCB-17

Principal Investigator: Sidney Pollack

Other Investigator: Edward Steers, Jr.

Man Years:

Total: 1.2
Professional: 1.1
Other: 0.1

Project Description:

Objectives: To isolate and characterize the structural proteins from trichocysts of Paramecium aurelia; to compare the proteins found in wild type with those found in various mutant strains; to gain an understanding of some of the processes associated with the development of this cellular organelle.

Methods Employed: Paramecia were grown in 5 gallon carboys (Steers, E., Jr., PNAS 48: 867, 1962) and also in 400 liter batches (Steers, E., Jr., Biochemistry 4: 1896, 1965). Undischarged trichocysts were obtained by suspending the paramecia in solutions of EDTA, disrupting them by homogenization, and then passing the homogenate through Sephadex G-200. Discharged trichocysts were obtained either by the use of dimethylsulfoxide and ethanol (Steers, E., Jr. et al., Exp. Cell Research 57: 392, 1969) or by other methods using EDTA and ATP. Trichocysts were solubilized by various agents. Polyacrylamide electrophoresis and column chromatography were performed on the solubilized proteins.

Major Findings: Wild type trichocysts can be solubilized in acid, base, and in denaturing agents such as guanidine hydrochloride, sodium dodecyl sulfate, lithium iodosalicylate. Analysis of the solubilized trichocysts by acrylamide disc electrophoresis in gels containing sodium dodecyl sulfate yielded results comparable to those obtained from trichocysts solubilized by SDS alone, namely, 2 bands with estimated molecular weights of 17,000 and 36,000. Modification procedures such as reduction and alkylation followed by maleylation or maleylation alone will also put this organelle into solution.

Employing these procedures, a more detailed analysis of trichocyst proteins is now being undertaken. This work will then be extended to the mutants.

Significance to Biomedical Research: This work represents a continuation of our objectives to characterize structural proteins related to the cell surface and cell surface peoperties using a specialized system in microorganisms. The analysis of genetic mutants will hopefully be useful in elucidating some of the problems of protein assembly into a cellular organelle.

Proposed Course of Project: Following an analysis of wild type and mutant structural proteins of trichocysts, we hope to obtain some information on the types of genetic lesions found in the mutants and thus to find out more about how this organelle, the trichocyst, develops.

Serial No. NIAMD-LCB-14
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Structure of the low density lipoprotein particle from human serum.

Previous Serial Number: NONE

Principal Investigator: Harvey Pollard

Man Years:

Total: 0.8
Professional: 0.8

Project Description:

Objectives:

1) Develop a quanitative description of the quaternary structure of low density lipoprotein (LDL).

2) Determine the chemical and physical basis for lipid-protein-interactions in LDL.

Methods Employed:

1) LDL was prepared from human plasma by flotation between d 1.019 and d 1.063 using sucrose to modify density of solvent.

2) Purity was ascertained on the basis of immunological and physical criteria.

3) Lipid depleted derivatives of LDL were prepared by solvent extraction processes, ranging from ether to chloroform/methanol mixtures.

4) Fluorescence and circular dichroism measurements of LDL derivatives under various conditions were performed.

5) Lipids were determined by quantitative assays of cholesterol and phospholipids, and thin layer chromatography was employed on a qualitative basis.

6) Electron microscopic images of LDL were scanned with an Isodensitracer and quantitative optical density maps were obtained.

Major Findings:

1) Removal of all neutral lipids from LDL resulted in a 75% loss of ordered structure relative to that in the native conformation. The derivative could not be further denatured by elevated pH.

2) Removal of all but 10% of the cholesterol and cholesterol ester

from LDL resulted in a particle with disordered quaternary structure, and with an only slightly decreased amount of secondary structure. Elevated pH resulted in a reversible denaturation, as measured by circular dichroism (CD). Intact LDL could not be denatured even at pH 12.5.

3. I conclude that most of the neutral lipid has structural significance for the LDL protein component, but that only a small amount is necessary to maintain the native structure at neutral pH. The prompt loss of much ordered structure upon removal of all neutral lipids suggests that a cooperative interaction involving protein, cholesterol and phospholipid may be occurring.

4. The spatial distribution of protein subunits in native LDL was found to be quantitatively distributed on the vertices of a 200 Å dodecahedron. A three-dimensional map of the twenty subunits delineated a central cavity where the neutral lipids apparently reside in native LDL. Removal of all neutral lipids resulted in a disordered quaternary structure.

Significance to Biomedical Research:

1) LDL is central to lipid transport and atherosclerosis in humans. A better understanding of function may be derived from our new knowledge of its structure.

2) The mechanism of transfer of neutral lipid, and the structural consequences of this transfer, may be approached through these studies on removal of neutral lipid *in vivo*.

3) Knowledge of the structure of the LDL particle may suggest ideas for homologous structures in biomembranes.

Proposed Course of Project:

1) I plan to make higher resolution reconstructions using Fourier analysis to average together many particle structures.

2) Reassembly of the native LDL from delipidated species will be attempted.

3) The chemical basis of cholesterol and phospholipid association with LDL will be examined by specific analysis of lipid binding by the isolated apo protein.

Serial No. NIAMD-LCB-15
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Interaction between the first enzyme for histidine biosynthesis and His-tRNA.

Previous Serial Number: NIAMD-LCB-18

Principal Investigators: John S. Kovach
Francesco Blasi (Univ. of Naples, Italy)
Robert Barton (NIAMD:LBM)
Robert F. Goldberger

Man Years:

Total: 1
Professional: 0.8
Other: 0.2

Project Description:

Objectives: On the basis of our previous work on the kinetics of derepression of the histidine enzymes we postulated that the first enzyme for histidine biosynthesis plays a previously unrecognized role in repression of the histidine system in Salmonella typhimurium. Because it has been known for some time that His-tRNA is required for repression of the histidine system, it appeared to us that one test of our hypothesis is to determine whether the first enzyme for histidine biosynthesis interacts with His-tRNA.

Methods Employed: The first enzyme for histidine biosynthesis (G enzyme) was purified on a large scale from extracts of Salmonella typhimurium which had been grown under conditions in which the histidine enzymes are derepressed. His-tRNA was isolated from S. typhimurium by standard procedures. Sephadex columns were set up and tested with known interacting macromolecular systems for use in experiments of the type described by Hummel and Dryer. Another technique for testing the interaction of G enzyme with His-tRNA, based on the nitrocellulose filter assay of Yarus and Berg, has been developed. Binding experiments done on Sepharose columns and nitrocellulose filters demonstrated that the G enzyme has a high affinity for His-tRNA in vitro (dissociation constant approximately 10^{-9} M). Comparing the binding of ^{3}H-His-tRNA with that of tRNA aminoacylated with other ^{3}H-amino acids disclosed that the binding of the histidyl species of tRNA is favored over that of other species and is dependent upon magnesium-ion concentration. In addition, we found that His-tRNA binds at a site on the enzyme distinct from the catalytic

site and the feedback-sensitive site; that the substrates of the enzyme inhibit the binding of His-tRNA, whereas histidine does not do so; that, once a complex has been formed between the enzyme and His-tRNA, the substrates of the enzyme decrease the stability of the complex, whereas histidine is without effect; and that purified enzyme which has a defect in its inhibition by histidine (produced by mutation) displays an altered ability to bind His-tRNA, a finding which may reflect the fact that mutants producing such a defective enzyme display an alteration of the repression process.

Significance to Biomedical Research: The finding that His-tRNA, a macromolecule known to be required for repression of the histidine system, binds with a very high affinity to the first enzyme of the histidine biosynthetic pathway, may be an important clue to understanding the mechanisms which regulate biosynthetic metabolic pathways.

Proposed Course of Project: We will seek conditions under which the enzyme displays a higher degree of specificity for the histidyl species of tRNA.

Publications:

Kovach, J.S., Phang, J.M., Blasi, F., Barton, R.W., Ballesteros-Olmo, A., and Goldberger, R.F.: Interaction between histidyl transfer ribonucleic acid and the first enzyme for histidine biosynthesis of Salmonella typhimurium. J. Bacteriol. 104: 787-792, 1970.

Blasi, F., Barton, R.W., Kovach, J.S., and Goldberger, R.F.: Interaction between the first enzyme for histidine biosynthesis and histidyl transfer ribonucleic acid. J. Bacteriol. 106: 000-000, 1971.

Martin, R.G., Berberich, M.A., Ames, B.N., Davis, W.W., Goldberger, R.F., and Yourno, J.D.: Enzymes and intermediates of histidine biosynthesis in Salmonella typhimurium. In Tabor (editor), Methods in Enzymology, Academic Press, New York, Vo. XVIIB, 1971.

Serial No. NIAMD-LCB-16
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The nature of the effect of alterations in the first structural gene of the histidine operon on repression of the operon.

Previous Serial Number: NIAMD-LCB-23

Principal Investigators: John S. Kovach
Tikvah Vogel

Other Investigator: Robert F. Goldberger

Man Years

Total: 0.5
Professional: 0.2
Other: 0.3

Project Description:

Objectives: On the basis of previous studies on the kinetics of repression of the histidine operon in Salmonella typhimurium, we suggested that the first enzyme for histidine biosynthesis (G enzyme) plays a previously unrecognized role in control of the operon. Subsequent studies showed that organisms producing a feedback resistant G enzyme could not be repressed by triazolalanine (TRA), an analogue of histidine which does repress organisms producing wild type G enzyme. We wanted to determine whether this effect of the G enzyme on repression is cis or trans, dominant or recessive.

Methods Employed: An episome bearing a wild type G gene was introduced into a feedback resistant strain. The merodiploid was tested for its ability to be repressed by TRA. The proper strains were constructed for selection of an episome bearing a G gene with a mutation to feedback resistance.

Major Findings: Whereas the feedback resistant strain itself was not repressible by TRA, the episome-bearing strain was repressible by TRA. This finding indicates that the effect of the G enzyme is trans. We therefore conclude that the G enzyme exerts its effect on the repression process not by acting locally, at its site of synthesis, but by acting as a freely diffusible gene product, as expected for a repressor.

Significance to Biomedical Research: The fact that the first enzyme of a biosynthetic pathway has a regulatory effect on its own synthesis and on the

synthesis of those enzymes contained in the same operon may be important to the elucidation of the mechanisms controlling biosynthetic pathways in bacterial systems.

Proposed Course of Project: Because episomal genes are represented by more copies per cell than are chromosomal genes, the reciprocal experiment must be done to determine if the trans effect is dominant or recessive. An episome bearing a mutation to feedback resistance will be isolated. This episome will be put into strains producing wild type G enzyme. The resulting merodiploids will be tested for repressibility by TRA.

Serial No. NIAMD-LCB-17
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Isolation of a high frequency transducing phage for the histidine operon in E. coli.

Previous Serial Number: NIAMD-LCB-22

Principal Investigator: Francesco Blasi (Univ. of Naples, Italy)

Other Investigator: Robert F. Goldberger

Man Years:

Total: 0.3
Professional: 0.1
Other: 0.2

Project Description:

Objectives: The isolation of a specialized transducing phage for the histidine operon would be of great importance for examining the mechanisms involved in regulation of the histidine system. No such phage is available at present. In E. coli, the chromosomal location of the histidine operon is far removed from the attachment sites of the temperate phages lambda and Ø80. Using the approach of Beckwith, we are attempting to transpose the histidine operon to a site close to the attachment site of Ø80 and then isolate a phage carrying the entire histidine operon in place of some of its own genes.

Methods Employed: An episome has been constructed which carries, in addition to the histidine operon, a temperature sensitive mutation affecting the autonomous replication of the episome. This episome has been introduced into a deletion strain lacking the entire histidine operon. A technique was then used which allows the selection of those strains which have integrated the episome at or near the Ø80 attachment site.

Major Findings: Several strains in which the entire histidine operon has been transposed to the Ø80 attachment site have been isolated.

Significance to Biomedical Research: It is hoped that this study will lay the groundwork for further studies on regulation of gene expression in bacterial systems.

Proposed Course of Project: Terminated (Dr. Blasi is continuing this project in Naples).

Serial No. NIAMD-LCB-18
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Purification of $tRNA^{His}$ and of the histidyl species of aminoacyl-tRNA synthetase.

Previous Serial Number: NIAMD-LCB-19

Principal Investigator: Francesco Blasi (University of Naples, Italy)

Other Investigator: Robert Goldberger

Man Years:

Total: 0.3
Professional: 0.3

Project Description:

Objectives: In conjunction with other studies on regulation of the histidine system in Salmonella typhimurium, we have developed an in vitro system for investigating the interaction between the first enzyme for histidine biosynthesis (G enzyme) and His-tRNA (see project #NIAMD-LCB-15). In order to use purified components in this assay system, we have purified the G enzyme and are attempting to isolate pure $tRNA^{His}$ by affinity chromatography.

Methods Employed: The histidyl species of aminoacyl synthetase was purified serendipitously during the purification of G enzyme. This highly purified histidyl-tRNA-synthetase was covalently bound to Sepharose. A mixture containing all species of tRNA, aminoacylated with H^3-histidine, was passed through the column. Although most of the tRNA (O.D. 260) could be washed through the column, all the radioactivity was retained. The radioactivity was released from the column by washing with 1.5 M NaCl in acetic acid, pH 3.0.

Major findings: Approximately 15% of the radioactivity eluted from the column was associated with tRNA. This material could be aminoacylated with histidine to a much greater extent than with other amino acids, indicating a significant degree of purification of $tRNA^{His}$. However, further purification was not accomplished upon repeated passage through the column.

Significance to Biomedical Research: This method of tRNA purification has provided partially purified preparations of $tRNA^{His}$ for use in our studies on regulation of the histidine system and may prove to be a method generally useful for the isolation of other species of tRNA.

Proposed Course of Project: Terminated.

Serial No. NIAMD-LCB-19
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effect of histidine on the enzyme which catalyzes the first step of histidine biosynthesis in Salmonella typhimurium.

Previous Serial Number: NONE

Principal Investigator: Francesco Blasi (University of Naples, Italy)
Robert Goldberger
S.M. Aloj (NIAMD:CEB)

Man Years:

Total: 0.2
Professional: 0.2

Project Description:

Objectives: The enzyme, phosphoribosyltransferase, which catalyzes the first step of the pathway for histidine biosynthesis in Salmonella typhimurium, is inhibited by the end product of the pathway, histidine. This project was undertaken to elucidate the mechanism of this inhibition.

Methods Employed: Phosphoribosyltransferase was purified from wild type Salmonella typhimurium and from a derivative of this strain in which a mutation in the first gene of the histidine operon leads to the production of a phosphoribosyltransferase which is not sensitive to inhibition by histidine. Using these two enzymes, we carried out studies involving spectrophotometric titration of phenolic hydroxyl groups, solvent perturbation difference spectroscopy, determination of tryptophan and tyrosine content, ultraviolet spectrofluorometry, and circular dichroic spectroscopy. All studies were done on the enzyme preparations in the absence of histidine and in the presence of histidine at various concentrations to assess the effect of histidine on the conformation of the proteins.

Major Findings: (1) In the presence of histidine the enzyme displays a positive ultraviolet difference spectrum. Analysis of this effect by the solvent perturbation method indicates a burying of chromophores upon addition of histidine. Spectrophotometric titration of the ionization of tyrosyl hydroxyl groups discloses that histidine prevents ionization of 12 tyrosyl residues in the enzyme. (2) Addition of histidine to the enzyme causes a shift in the fluorescence emission spectrum, the extent of which is dependent upon histidine concentration. Titration of the shift with histidine reveals a non-

linear behavior, suggesting a cooperativity in this effect of histidine. (3) Addition of histidine to the enzyme does not significantly alter the circular dichroic spectrum of the enzyme. This indicates that histidine does not cause a significant change in the secondary structure of the enzyme. (4) Histidine-insensitive enzyme, isolated from a feedback-resistant mutant, does not show the change in fluorescence which is produced by histidine in the wild type enzyme. The mutant enzyme does, however, bind histidine, judging from the fact that it, like the wild type enzyme, is protected by histidine against denaturation by urea. We conclude that inhibition of phosphoribosyltransferase by histidine is brought about by a change in the conformation of the enzyme. Although phosphoribosyltransferase from a feedback-resistant mutant is able to bind histidine, it is unable to undergo the conformational change characteristic of the wild type enzyme. Thus, the failure of the mutant enzyme to be inhibited by histidine is probably due to an alteration of its structure which does not permit a conformational change to occur when it binds histidine.

Significance to Biomedical Research: Feedback inhibition is one of the most important mechanisms by which the biosynthesis of macromolecules is regulated in bacterial and in animal cells. Deeper understanding of this phenomenon at the molecular level leads to a deeper understanding of the physiological basis for cellular regulation.

Proposed Course of Project: Terminated.

Publication:

Blasi, F., Aloj, S.M., and Goldberger, R.F.: Effect of histidine on the enzyme which catalyzes the first step of histidine biosynthesis in *Salmonella typhimurium*. Biochemistry 10: 1409-1417, 1971.

Serial No. NIAMD-LCB-20
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mutations in the first structural gene of the histidine operon which alter regulation of the operon.

Previous Serial Number: NIAMD-LCB-21

Principal Investigator: John S. Kovach

Man Years:

Total: 1.6
Professional: 0.8
Other: 0.8

Project Description:

Objectives: On the basis of previous work done on the kinetics of repression of the histidine operon in Salmonella typhimurium, we suggested that the first enzyme for histidine biosynthesis plays a previously unrecognized role in control of the histidine operon. A prediction of this hypothesis is that certain mutations in the first structural gene (G gene) of the operon would lead to alterations in the structure of the first enzyme which affect regulation of the histidine operon. Although a number of mutations affecting the catalytic function of this enzyme are known, none of these affect the process of repression. I am attempting to find mutations in the first structural gene which do alter the repression process.

Methods Employed: Two selection procedures have been used. As reported last year, initial attempts to obtain mutations in the G gene involved selecting histidine auxotrophs from a strain containing two normally functioning genes for each of the histidine biosynthetic enzymes except for the G gene which is represented only once. Theoretically, all histidine auxotrophs derived from this strain should have mutations in the G gene. Although this method has led to the isolation of several strains bearing mutations in the G gene, this has been an extremely rare event, and none of these auxotrophs shows any change in the process of repression.

To be certain that every organism selected for study contains a new mutation in G gene another procedure has been tried recently. A large number of prototrophic revertants of several different complete G mutants are being examined for alterations in regulation of the histidine operon. It is hoped that by using a variety of mutagenic agents to induce reversion of stable auxotrophic mutations at different points along the G cistron, one or more sites

may be found which when altered lead to changes in repression control of the operon.

Major Findings: Thus far one revertant of a stable G auxotroph has been demonstrated to have altered repression control of the histidine operon. Fifteen other revertants of the same suxotroph also appear to have altered regulatory properties. This has not been rigorously proven, as yet. However, of approximately 5000 revertants of a different G auxotroph, none with altered regulatory properties has been found.

Significance to Biomedical Research: If alterations in the first enzyme for histidine biosynthesis which affect repression of the histidine operon are found, they will constitute proof of our hypothesis, that a biosynthetic enzyme may have regulatory properties which allow it to control its own synthesis as well as the synthesis of the other biosynthetic enzymes.

Proposed Course of Project: Initally, attempts will be made to isolate more revertants with altered regulatory properties. It will then be necessary to demonstrate that the new mutation affecting regulation is linked to the histidine operon and is not in one of the five genes unlinked to the operon which are known to affect regulation.

ANNUAL REPORT SUMMARY

LABORATORY OF BIOCHEMICAL PHARMACOLOGY

A. *PROTEIN STRUCTURE AND MECHANISM OF ENZYME ACTION*

1. Tryptophan Synthetase

Tryptophan synthetase, an enzyme complex of two different proteins, A and B, is being studied in order to learn more about interaction and control in multienzyme systems. The B protein contains a cofactor, pyridoxal phosphate, which is being used as an optical and chemical probe in studies of the mechanism of the active site.

Recently completed studies on chemical modification of the sulfhydryl residues of the B protein have given information on the active site and interaction sites of tryptophan synthetase. Tryptic peptides containing specifically modified sulfhydryl residues from the B protein of two different bacterial species are being isolated and compared. (Dr. Edith W. Miles)

Three analogs of the cofactor, pyridoxal 5'-phosphate, have been synthesized for the first time: α^5-pyridoxal chloride, α^5-pyridoxal methylchloride, and α^5-pyridoxal ethylchloride. All three analogs irreversibly inactivate the apo B protein. α^5-Pyridoxal methylchloride reacts stoichiometrically and covalently at the active site of the apo B protein under optimal conditions. Current studies to identify the amino acid residue and tryptic peptide which are alkylated by the analog should lead to further understanding of the cofactor binding site. (Drs. Edith W. Miles and Jerry B. Gin)

Substrate analogs which can be utilized or which inactivate the B protein are being investigated. Several new β-elimination and β-addition or β-replacement reactions of the B protein with S-methyl-L-cysteine and O-methyl-L-serine have been discovered. These give further information on the mechanism and substrate specificity of tryptophan synthetase and allow useful comparisons with the mechanism of a related degradative enzyme, tryptophanase. (Drs. Edith W. Miles and Hidehiko Kumagai)

2. Histidine Ammonia-Lyase

Subunit Structure -- Histidine ammonia-lyase (MW 215,000) was previously shown to be composed of 4 subunits similar in size (53,000). The enzyme, after treatment with thiols, contains 16 half cystine residues; 4 of these are reactive towards N-ethylmaleimide or Ellman's reagent, and 12 are buried cysteinyl residues. After carboxymethylation of the 4 reactive cysteines with ^{14}C iodoacetate, followed by carboxymethylation of the remaining cysteines with ^{12}C iodoacetate in 8 M urea, a tryptic peptide map of the enzyme showed a single ^{14}C labeled peptide of 18 amino acid residues. These data suggest that each of the 4 subunits contains an identical peptide which includes the reactive cysteinyl residue. (Drs. Claude B. Klee and Jules A. Gladner)

Metal Ions - Sulfhydryl Groups Relationship -- Histidine ammonia-lyase has been obtained free of metal ions and shown to be dependent on the addition of

Mn^{++}, Zn^{++}, or Cd^{++} for enzymatic activity. Both the oxidized (no free -SH groups) and the reduced (4 free -SH groups) forms of the enzyme are dependent on a metal. The increased enzymatic activity of the reduced enzyme is due to an increased affinity for metal ions. In the presence of metal ions, the 4 -SH groups of the reduced enzyme are no longer titratable by Ellman's reagent. The presence of substrate facilitates the binding of the metal to the enzyme. These data suggest that the physiologically active form of the enzyme is a metal mercaptide. (Dr. Claude B. Klee)

3. Lactose Synthetase

α Lactalbumin modifies the activity of the A protein of lactose synthetase by altering its affinity for acceptor sugars. Purified A protein from bovine milk is a single chain glycoprotein which can be shown to form a 1 : 1 complex with α lactalbumin, but only in the presence of substrate; thus, lactose synthetase can be considered to be an enzyme composed of 2 dissimilar subunits which are associated in a transient fashion when the enzyme is functioning. (Drs. Werner Klee and Claude B. Klee)

4. Glycyl tRNA Synthetase

The activation and regulation of glycyl tRNA synthetase, an enzyme with a role in protein synthesis, is being studied. The past year was spent perfecting a test for a specific regulator substance, and beginning the development of a procedure to purify it. (Dr. Simon Black and Mrs. Blondel Hazel)

5. Structure-Function Studies of Hydroxypyruvate Reductases

The subunit structure, kinetics, and sulfhydryl properties of spinach glyoxylate reductase have been compared to analogous properties of several bacterial hydroxypyruvate utilizing enzymes. It is clear that the catalytic mechanism is independent of subunit structure. An active site peptide has been isolated and demonstrated to be effectively identical in amino acid composition in three of these reductases and very similar in its composition to glyceraldehyde 3-phosphate dehydrogenase. Anion regulation has been related to a specific anion site adjacent to the carbonyl site on the active center and to conformational states of the secondary structure. Specificity appears to be related to secondary structure rather than differences in this active site peptide. Subunit reassociation has been demonstrated and characterized. It has been shown to be independent of disulfide bond formation, but dependent on subunit conformation. The kinetic mechanism of these enzymes has been investigated. (Drs. Leonard D. Kohn and John M. Utting)

6. Relationships of Thyrotropin and Exophthalmogenic Factor

Studies of the relationship of exophthalmic factor to TSH have indicated that homogeneous bovine glycoproteins exist which contain both activities. These have similar amino acid and carbohydrate compositions and terminally positioned galactose residues. One is uniquely sensitive to pepsin, losing all its TSH but none of its EPS activity. One glycopeptide produced contains all the EPS activity; its molecular weight is 20,000. It appears to be

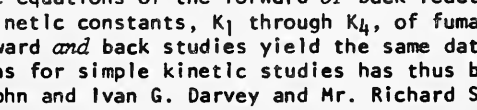
composed of a major portion of the B subunit of TSH but only a small portion (MWT 6,000) of the α subunit. The glycopeptide is active in harderian gland mucopolysaccharide biosynthesis, SO_4 activation being one of the earliest *in vivo* effects. Human TSH is similarly affected by partial pepsin digestion, preserving its EPS action but losing its TSH function. Tumor TSH has an EPS action unmasked during pepsin digestion. (Drs. Leonard D. Kohn and Roger J. Winand)

7. Studies of Integrated Rate Equations

Integrated rate equations of the forward *or* back reaction have been shown to yield all the kinetic constants, K_1 through K_4, of fumarase. Previous work requiring *both* forward *and* back studies yield the same data. The validity of integrated equations for simple kinetic studies has thus been demonstrated. (Drs. Leonard D. Kohn and Ivan G. Darvey and Mr. Richard Shrager)

B. *COMPLEX CARBOHYDRATE*

A new hexasaccharide has been isolated from human milk and its structure determined:



This sugar is especially interesting for two reasons: (1) its branched structure is identical to the structure proposed by Kabat [*cf., Proc. Nat. Acad. Sci.*, 61, 1470 (1968)] for the main chain of blood group determinants and provides proof that his proposal is correct; and (2) the sugar is a potent hapten inhibitor of anti-I sera, suggesting that this structure may be an immunizing antigen in auto-immune hemolytic anemias, many of which result from anti-I cold agglutinins. (Drs. Victor Ginsburg, Akira Kobata, Catherine A. Hickey, and Teresa Sawicka)

C. *CELL SURFACES*

Treatment of *E. coli* with EDTA causes release of half of the surface lipopolysaccharide (LPS). To determine the location of the releasable LPS relative to the remainder, the adsorption of ε15 phage to *Salmonella anatum* was measured, since this adsorption is known to be dependent on LPS. The results show that at low multiplicities the rate and extent of adsorption is equal in EDTA-treated and control bacteria, suggesting that there is no barrier to the accessibility of phage to the remaining LPS. At high multiplicities, only half as many phage attach to EDTA-treated as to control bacteria. These results indicate that the EDTA treatment does not expose a new, previous inaccessible LPS layer, but rather that the two LPS fractions are equivalently accessible to phage. It is thus possible to postulate that they are equally

external in their location. (Drs. Loretta Leive and David A. Lawrence)

Although under normal growth conditions approximately half of the total LPS of *E. coli* is releasable by EDTA, current experiments indicate that if the concentration of divalent cations present during growth is greatly increased, the fraction of LPS subsequently releasable by EDTA can also be increased. These results are consistent with previous isotopic evidence that the releasable LPS is in metabolic equilibrium with the remainder. One may postulate the following equation: $LPS\text{-}X^{+} \rightleftarrows LPS\text{-}Me^{++}$, where X is an unknown cationic grouping that cannot be chelated by EDTA. (Drs. Loretta Leive and David C. Morrison)

A new method for extracting and purifying LPS has been devised, employing treatment of cells with aqueous butanol-1, followed by pronase digestion and ultrafiltration. The resultant LPS is at least 95% pure and shows properties different from that of LPS isolated by conventional means. In particular, the density of material prepared by this method is less than that of LPS prepared by the standard (phenol) extraction, which suggests a higher lipid content. Nevertheless, the usual methods for assaying the lipid moiety of LPS (lipid A) reveal only 10-15% of this fraction in material isolated by the new method as in material isolated by phenol extraction. The reasons for these contradictory results are under investigation. Since the butanol-extracted LPS can be modified by the action of phenol to resemble phenol-extracted LPS in density, it is possible that the butanol extraction retains the LPS in a more nearly native configuration. (Drs. David C. Morrison and Loretta Leive)

D. *POLYAMINES*

Spermidine biosynthesis in *E. coli* is carried out by an enzyme that transfers the propylamine moiety from decarboxylated adenosylmethionine to 1,4-diaminobutane. This transferase has been purified about 2,000-fold to homogeneity. The enzyme has a molecular weight of about 73,000; it is composed of two apparently homogeneous subunits, of 35,000 molecular weight. The enzyme has no demonstrable requirement for added cofactors. The same enzyme carries out the synthesis of spermine; however, the pH optimum is much higher, and the K_m is much greater. (Drs. William H. Bowman, Herbert Tabor, and Celia White Tabor)

The glutathionyl-spermidine derivative in *E. coli* is formed when full grown cultures are incubated anaerobically at pH of 6.2 or lower. In strictly anaerobic conditions the compound can be formed rapidly, and essentially all of the spermidine is converted to the derivative. About half of the glutathione in *E. coli* is also converted to this derivative. On dilution into fresh medium and on aeration, glutathionyl-spermidine is hydrolyzed to form spermidine and glutathione. An enzyme which carries out this hydrolysis has been partially purified from *E. coli*. (Drs. Herbert Tabor and Celia White Tabor)

Acetylation of spermidine occurs when *E. coli* is stored in the cold. Studies on changes in the ribosomes of *E. coli* during storage in the cold

have shown that the ribosomal changes are not dependent on the changes in the spermidine content. (Dr. Celia White Tabor and Mrs. Patricia Deal Kellogg)

E. *PROTEIN AND NUCLEIC ACID*

1. Nucleic Acid Metabolism

The DNA-polymerase induced by T4 bacteriophage has both polymerase and exonuclease activities. We have previously shown that after infection with some amber mutants in this gene, there is no polymerase activity, but there is an exonuclease activity similar to that of the wild type enzyme. We have now demonstrated that the *am* B22 mutant nuclease is an amber fragment of the wild type protein by cochromatography of the tryptic peptides of the two proteins, after differential labeling with radioactive iodoacetic acid. Both proteins are single polypeptide chains, and the molecular weight of the mutant by gel electrophoresis with SDS and mercaptoethanol and by high speed equilibrium sedimentation (about 82,000) is consistent with its position in the gene. The mutant and wild type enzymes degrade from the 3' hydroxyl chain terminus randomly rather than processively (one chain at a time). Although the degradation is random, polymers of intermediate size do not accumulate and the 5' end of a chain 200 nucleotides long is degraded without a lag because the intermediate size chains are degraded at a faster rate than the initial substrate. The mutant enzyme differs from the wild type in its markedly decreased affinity for DNA, oligonucleotides, and deoxynucleoside triphosphates, and in its inability to synthesize DNA and to carry out the terminal addition of a single nucleotide.

In studies designed to elucidate the role of the exonuclease during DNA synthesis, we have confirmed that the degradation of template DNA is inhibited by deoxynucleoside triphosphates and have shown, in contrast, that the degradation of newly synthesized DNA does occur under these conditions since deoxynucleoside triphosphates are converted into monophosphates. This conversion continues after incorporation has reached a constant level and is dependent upon polymerization since it requires a 3' hydroxyl primer, a template, and a nucleotide which can be incorporated with that template. The *am* B22 mutant enzyme which cannot incorporate deoxynucleoside triphosphates, cannot convert them into monophosphates. (Drs. Nancy G. Nossal and Michael S. Hershfield)

2. Protein Synthesis

Studies in vivo -- We have continued our work on the temperature sensitive mutant of *E. coli* which was described in the previous Annual Report. We have established that inability of the cell to accumulate protein at 42° is due to a decrease in synthetic ability and not due to a massive increase in protein degradation. This result, coupled with our earlier data on the stability of polysomes at 42° and the persistence (for a short time at least) of RNA and DNA synthesis at 42°, suggests that the lesion in this mutant may reside in a heretofore unrecognized part of the cell's protein synthesizing apparatus. (Dr. Anthony V. Furano)

Studies in vitro -- We have completed our studies on the effect of

oxidized glutathione (GSSG) on ribosomes and have made the following observations: (1) Prolonged (24 hour) anaerobic incubation at 37° of ribosomes in the *absence* of GSSG leads to extensive degradation of all of the ribosomal RNA to pieces with a sedimentation in sucrose gradients of about 5S; (2) Such ribosomes are essentially fully active when assayed for poly U directed polyphenylalanine synthesis; and (3) if the above incubation includes GSSG (0.05 M), then all of the 70S ribosomes are dissociated into 30S and 50S subunits *and* the RNA is degraded in a manner different from ribosomes incubated in the absence of GSSG. We showed that this different pattern is not due to the dissociation *per se* but due to the fact that the GS- moiety of GSSG is bound to the ribosomal protein of both the 30S and 50S subunits. These observations suggest: (a) intact ribosomal RNA is not required for ribosomal function; (b) there is some structural periodicity to the ribosome so as to allow the nucleolytic production of 5S pieces; and (c) the GSSG *via* its interaction with ribosomal protein has altered the structure of the ribosome as reflected in a different manner of RNA degration.

These studies also have confirmed and extended earlier reports from this laboratory (and that of Traut and Haenni) that ribosomal SH's are involved in subunit association into 70S ribosomes, the binding of phe tRNA by the 30S subunit, but not the binding of poly U by the 30S subunit. (Drs. Anthony V. Furano and Maureen I. Harris)

3. Vaccinia tRNA

Uridine-labeled HeLa tRNA, when chromatographed on benzoylated diethylaminoethyl cellulose (BD-cellulose), elutes in two peaks, A and B, which contain 53% and 47% of the total labeled tRNA, respectively. The ratio of peak A to peak B is about 1.15. Experiments with methyl-labeled tRNA suggest that the source of this chromatographic separation depends on the methyl groups found in the bases of the tRNA species. The tRNA species that elute as peak A are relatively methyl-rich and contain 70% of the total methyl-labeled tRNA while those molecules that elute later at the higher salt as peak B are relatively methyl-poor and contain 30% of the total methyl-labeled tRNA.

Infection of HeLa cells with vaccinia for 8 hours results in a significant change in the elution pattern of uridine-labeled tRNA. About 70% of the uridine-labeled tRNA is found to elute in the methyl-rich peak A while 30% is found to elute in the methyl-poor peak B. The ratio of uridine counts in peak A to peak B is about 2.20 compared to the 1.15 found in uninfected cells. Since preexisting tRNA can be shown to be unaffected by vaccinia infection, it was concluded that in vaccinia infected cells there is a substantial relative increase in the synthesis of methylated tRNA compared to normal cells.

Thin layer chromatography of methylated bases has not yet shown any difference in the methylated base composition of HeLa and vaccinia tRNA. The same negative result is true for SV40 and monkey tRNA. However, it is possible that sequences containing methylated bases differ for viral and host tRNA. This is under investigation. (Dr. Michael Klagsbrun)

F. *TRAUMATIC SHOCK AND TRANSPLANTATION*

Experiments on the effect of environmental temperature in burned mice demonstrate that (1) 48 hour or shock mortality is significantly lowest in animals maintained at 25° C compared with 4°, 31°, and 37° C; and (2) late mortality is not significantly different in animals kept at 31° or 25°, in spite of the fact that nitrogen metabolism and body temperature is much less disturbed at the higher temperature. These results may have important clinical implications in the treatment of burned patients.

Experiments on the effect of traumatic and non-traumatic shock on skin allograft survival demonstrate that burn or tourniquet trauma significantly prolonged the survival time of first set grafts, regardless of whether the trauma was applied as early as 21 days before grafting or as late as 7 days postgraft. Daily intraperitoneal injections of *E. coli* lipopolysaccharide, a non-traumatic form of shock, significantly prolonged survival time of first set grafts, although not to the same extent as both types of traumatic shock. On the other hand, hyperosmolar shock one day prior to grafting had no effect on survival time of skin grafts. A common denominator that increased survival time of allografts was tissue damage. (Dr. Kehl Markley, Mrs. Elizabeth Smallman, and Mr. Steven W. Thornton)

The influence of protease inhibitors (phenylmethylsulfonyl fluoride, ovomucoid, and lima bean inhibitor) on the swelling following thermal and tourniquet trauma to the mouse tail was determined. A pronounced inhibition was observed in burn trauma but not in tourniquet, indicating that proteases are involved in the swelling of burn trauma. An apparatus was developed to measure swelling pressure of tissues following application of tourniquet and burn trauma to the rodent tail. (Dr. Sanford M. Rosenthal and Mr. Steven W. Thornton)

G. *TISSUE CULTURE STUDIES (GRANULOCYTES AND MACROPHAGES)*

A simple technique for the study of granulocytopoiesis *in vitro* was developed. Bone marrow cells of mice were grown in a simple medium without using the soft-agar and the colony-stimulating factors which were reported by other investigators to be essential for growth of bone marrow cells. Colonies of granulocytes were observed. The total number of granulocytes produced per colony was comparable to that reported by others. An interesting finding was the demonstration of a natural feeder cell which was observed to be related to the formation of the cell colony.

H. *MYCOBACTERIUM*

Solanocapsine, an alkaloid isolated by Dr. Y. Sato from *Solanum pseudocapsium*, commonly called Jerusalem cherry or Christmas cherry, showed bacteriostatic activity against *Mycobacterium lepraemurium* grown in macrophage cultures. Solanocapsine has a sterol ring structure and configuration. It is apparently the first sterol compound which exhibits an antibacterial

activity against an obligate intracellular microorganism. (Dr. Yao Teh Chang and Mrs. Roxanna L. Andersen)

The acid-fastness of *M. leprae* was eliminated after pyridine extraction, while that of *M. lepraemurium* remained unchanged. It seems reasonable that the pyridine extraction - Ziehl-Neelsen technique could be employed as a simple method for distinguishing these two non-cultivable mycobacteria. (Dr. Yao Teh Chang and Mrs. Roxanna L. Andersen)

I. *EVOLUTION*

A theory has been proposed and published to show that enzymes could have evolved by simple mutation and selection from random polypeptides that arose at the very beginning of organic evolution. (Dr. Simon Black)

Serial No. NIAMD-LBP-1
1. Biochemical Pharmacology
2. Biochemistry of Amino
3. Acids

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Biochemistry of Sulfur-Containing Compounds

Previous Serial Number: Same

Principal Investigator: Dr. Simon Black

Other Investigator: Mrs. Blondel Hazel

Cooperating Units: None

Man Years

Total:	4.2
Professional:	2.0
Others:	2.2

Project Description:

Objectives:

A long term objective is discovery of enzymatic and regulatory mechanisms involved in the synthesis of constituents of living tissue, particularly of proteins.

Methods Employed:

Chemical and enzymatic techniques involving the use of spectrophotometry and radioactive substances are evolved to meet the needs of special problems as they arise.

Major Findings:

I. Protein Synthesis. The glycyl-tRNA synthetase from yeast, an essential enzyme in protein biosynthesis, catalyzes the following reaction:

$$\text{Glycine} + \text{ATP} + \text{tRNA} \rightleftarrows \text{Glycyl-tRNA} + \text{AMP} + \text{PP}$$

It has been found previously that when this enzyme is tested in the presence of inorganic sulfide, its activity becomes completely dependent upon two activator-like substances, glutathione and an alkali-denatured protein. It

is our hypothesis that the denatured protein acts as a substitute for a specific activator present in the cell, and efforts have been directed to developing a test which will distinguish between this activator and non-specific ones. During the past year a practical test for such a specific substance has been developed, and development of procedures for its purification and characterization has been started.

II. Evolution. A theory has been conceived to show that enzymes could have evolved from simple polymers of amino acids, arranged in a random order.

Significance to Bio-medical Research and the Program of the Institute:

I. Protein Synthesis. Elucidation of the nature of the postulated activactor and its interaction with the enzyme would open to similar study the regulation of many other of the more complex anabolic reactions of the cell, which are thought to be related to the functions of the hormones.

II. Evolution. It is hoped that a plausible theory for the origin of living organisms, supported by observation and experiment, will throw light on the nature of fundamental chemical interactions in present-day cells, and that this will be useful in enlarging our understanding and control of disease processes.

Proposed Course:

I. Protein Synthesis. With a test for a specific activator now in hand, efforts will be made to isolate and identify this substance.

II. Evolution. Theoretical and experimental work are being pursued with a view to determining how the first proteins and nucleic acids arose in nature, and how the genetic code originated.

Honors and Awards

None

Publications:

Black, S.: Pre-cell evolution and the origin of enzymes. *Nature*, 226, 754 (1970).

Black, S.: A hypothesis and an experiment on the origin of the genetic coding process. *Biochem. Biophys. Res. Commun.*, in press.

Serial No. NIAMD-LBP-2
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Chemotherapy of Mouse Leprosy

Previous Serial Number: Same

Principal Investigator: Dr. Yao Teh Chang

Other Investigator: Mrs. Roxanna N. Andersen

Cooperating Unit: Leonard Wood Memorial (The American Leprosy Foundation)

Man Years

Total:	3.1
Professional:	2.0
Others:	1.1

Project Description:

Objectives:

The evaluation of therapeutic effectiveness of drugs in mouse leprosy. The tissue cultures of intracellular parasites.

Methods Employed:

Chemotherapeutic studies were performed in mice infected intraperitoneally with *Mycobacterium lepraemurium* or into food pads with *M. lepraemurium* and *M. leprae*. Screening of antibacterial drugs was also made in our macrophage--*M. lepraemurium* cell model. Further attempts to cultivate *M. leprae* were made using cultures of macrophages obtained from mice which had been thymectomized and irradiated with 950 γ. Bone marrow cells of mice were cultivated in Leighton tubes and attempts were made to adapt human marrow and blood macrophages to the same system. Smears of various strains of acid-fast organisms were extracted in fresh pyridine at 60° C and their acid-fastness was examined 3 to 24 hours later.

Major Findings:

Effect of pyridine extraction on the acid-fastness of M. leprae -- Campo-Aasen and Convit reported a histochemical method for identification and differentiation of two non-cultivable mycobacteria, *M. leprae* and *M. lepraemurium*.

Employing Baker's technique for the extraction (with pyridine) and staining (with acid hematein) of phospholipids, they observed that the phospholipid component of *M. leprae* was eliminated after pyridine extraction, while that of *M. lepraemurium* remained intact. This technique is, however, tedious and time consuming, and the dark blue color of the bacilli is not always distinctive. We employed pyridine extraction but substituted the Ziehl-Neelsen stain in place of acid hematein. It was observed that the acid-fastness of *M. leprae* was eliminated after pyridine extraction, while that of *M. lepraemurium* remained unchanged. It seems reasonable that the pyridine extraction - Ziehl-Neelsen technique could be employed as a simple method to differentiate the two non-cultivable mycobacteria, *M. leprae* and *M. lepraemurium*.

Individual variation of the growth of M. leprae in mouse foot pads -- Current studies on the quantitative anti-*M. leprae* evaluation of chemotherapeutic agents, vaccines, etc., have been based mainly on the counts of bacterial suspensions made of 4 pooled foot pads. From the literature one obtains the impression that this model represents a uniform infection, and findings from 4 animals at a time adequately reflect the trend of a larger population. Since controlled study on individual variations in the foot pads has not yet appeared in the literature, we performed this type of study in a group of 24 CBA/J mice, the strain which best exhibited a uniform intraperitoneal *M. lepraemurium* infection among a total of 14 inbred strains observed in our laboratory.

Marked individual variations were observed. 58.3% of animals showed bacterial counts of lower than 19×10^4, 33.4% of animals between 40×10^4 to 85×10^4, and 8.3% of animals above 1×10^6 organisms per foot pad. The highest count was 4.3×10^6 and the lowest only 9×10^3, a difference of about 480-fold. When the data was arranged randomly in groups of 4 mice, a difference of 13-fold was observed between the highest and lowest groups. Three drugs, DDS, B.663, and B.1912, were tested in these animals. The bacterial count of controls was 1.1×10^6, while those of the 3 treated groups ranged from 3.6×10^4 to 9×10^4. Although a definite difference was observed by the bacterial counts, the difference was not significant when treated statistically because of marked individual variations. This raised the question of reliability of the present foot pad screening technique for quantitative evaluation.

Antibacterial activity of solanocapsine against M. lepraemurium in macrophage cultures -- Solanocapsine, an alkaloid of *Solanum pseudocapsinem*, commonly called Jerusalem cherry or Christmas cherry, has been reported to have an antibacterial activity at 3 µg/ml against *M. tuberculosis in vitro*. It showed suppressive activity against *M. lepraemurium* in macrophage cultures in concentrations of 1, 3, 6, and 9 µg/ml. Solanocapsine has a sterol ring structure and configuration. It is apparently the first sterol compound which exhibits an antibacterial activity against an obligate intracellular microorganism. This drug was kindly supplied by Dr. Yoshio Sato of the Laboratory of Chemistry, NIAMD.

A simple technique in the study of granulocytopoiesis in vitro -- Bone marrow cells obtained from the femurs and tibia of mice were grown in our

medium used for growth of macrophages. The soft-agar and the colony stimulating factors which were reported by other investigators to be essential for growth of bone marrow cells were not used. In spite of this omission, many cell colonies were observed on the second day of cultivation. Various types of precursors of granulocytes were observed in the cell colonies. Large numbers of mature granulocytes were accumulated in the medium. The number of granulocytes produced per cell colony was comparable to that reported by other investigators. An interesting finding was the demonstration of a natural feeder cell which was observed to be related to the formation of the cell colony. With the present technique the process of granulopoiesis can be studied in stained preparations or under phase contrast microscopy as well as cinemicrography.

Growth of M. lepraemurium in foot pads of various inbred strains of mice -- Comparison studies were made on the growth rate of *M. lepraemurium* in the foot pads of a total of 16 inbred strains of mice. Preliminary results reveal that marked variations in the growth were observed not only among the different strains of mice but also among the individual animals of the same strain.

Studies on growth of M. leprae in macrophages obtained from thymectomized and irradiated mice -- Beginning in January, a monthly supply of 20 thymectomized and irradiated mice was kindly furnished by the Oak Ridge National Laboratory through arrangement of the Leprosy Panel of the United States-Japan Cooperative Medical Science Program. Macrophages obtained from the bone marrow and peritoneal cavity of these animals were infected with *M. leprae*. Tremendous *in vivo* growth of *M. leprae* has been observed in these immunosuppressed animals and it is hoped that the macrophages of these animals might remain in the suppressed state *in vitro* to facilitate the growth of *M. leprae* in cell cultures. These experiments are in progress.

Cultivation of bone marrow macrophages -- Mouse bone marrow macrophages can now be maintained in good condition for about 20 months. They appear as pure macrophages without contamination of fibroblast-like cells. Multiplication of macrophages was evidenced by constant sloughing of new cells into the culture medium. Serial subcultures were made by inoculating the discarded cells collected from the mother culture's medium. Despite our success in dealing with mouse macrophages, studies on the growth of human macrophages (blood and bone marrow) have been unsuccessful.

Growth pattern of M. lepraemurium in different type of macrophages -- Comparison on the growth pattern of *M. lepraemurium* in macrophages obtained from different sites revealed the following: (1) In peritoneal macrophages the organisms multiplied continuously, reaching the maximal stage in 6-7 weeks. At that time all macrophages were crammed with organisms resulting in destruction of the host cells. (2) In bone marrow macrophages multiplication of organisms was accompanied by simultaneous division of the host cells. Therefore, the infection was maintained in a stationary state in which about half of the cell population contained numerous organisms while the other half remained germ-free. (3) The growth of *M. lepraemurium* in bone marrow macrophages which had been kept in culture for 14 months showed the same pattern as in macrophages obtained directly from the mouse.

In a cooperative study with Dr. S. C. Chang of the Leonard Wood Memorial studies on ultrastructures of peritoneal cells and bone marrow cells are in progress.

Significance to Bio-medical Research and the Program of the Institute:

The successful growth of mouse bone marrow granulocytes in a simple culture medium furnishes a valuable model for studies of the process of granulocytopoiesis *in vitro*. Current cultivation technique involves a semi-gel system which requires histological section for detailed study of the cell colonies. With our system, the process of granulocytopoiesis could easily be observed either in stained slides or under phase-contrast microscopy. It should be noted that in the past *in vitro* studies on granulocytopoiesis, in normal or pathological conditions, have been handicapped chiefly by the lack of suitable cultivation methods.

The observation that marked individual variations on the growth of *M. leprae* in foot pads has appeared in a highly inbred strain of mice is of paramount importance at the present time. Current studies involving the foot pad model are based on counts of bacterial suspensions made from pooled foot pads of 4 animals. In our laboratory, we have observed not only marked individual variation in foot pad counts within a large group of animals, but also marked difference between counts from randomly arranged groups of 4 animals each. Unfortunately, many studies using this model have been made in several laboratories in the last decade, and the findings have been applied clinically resulting, according to some opinions, in possible chemotherapeutic and epidemiological misdirection. This indicates that a thorough study on the uniformity of growth of *M. leprae* in foot pads of untreated animals is urgently needed. Development of a model which would yield statistically significant results is needed before a quantitative evaluation of the growth of *M. leprae* in mice can be made.

Proposed Course:

Studies will be continued toward the development of techniques for cultivation of human macrophages, on the growth of *M. leprae* in cultures of macrophages obtained from thymectomized and irradiated mice, on the effect of pyridine extraction on the acid-fastness in various species of acid-fast organisms, on the screening of solanocapsine activity in mice infected with *M. leprae* and *M. lepraemurium*, on the activity of derivatives of solanocapsine in the macrophage-*M. lepraemurium* cell system, and on *in vitro* granulopoiesis and other cell development from the bone marrow.

Honors and Awards

None

Publications:

Chang, Y. T.: Effect of B.1912, a new riminophenazine derivative, in murine leprosy. *Int. J. Leprosy*, 38, 417-421 (1970).

Chang, Y. T., and Andersen, R. N.: Cultivation of mouse bone marrow cells. I. Growth of granulocytes. *J. Reticuloendo. Soc.*, in press.

Serial No. NIAMD-LBP-3
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in Traumatic Shock and Cellular Immunity

Previous Serial Number: Same

Principal Investigator: Dr. Kehl Markley

Other Investigators: Dr. Sanford M. Rosenthal, Mrs. Elizabeth T. Smallman, and Mr. Steven W. Thornton

Cooperating Units: None

Man Years

Total:	4.0
Professional:	3.5
Other:	0.5

Project Description:

Objectives:

(1) To study the effects of environmental temperature on experimental burn mortality.

(2) To investigate the mechanism of prolongation of skin graft survival after burn trauma.

(3) To study the effects of protease inhibitors on swelling following burn and tourniquet trauma.

Methods Employed:

(1) Mice were given a 2/3 body surface area burn at 70° C for 5-8 seconds and placed at environmental temperatures of 4°, 25°, 31°, and 37° C. Mortality was recorded for 48 hours for shock mortality and from 2-21 days for late mortality. Measurements were made of body weight, food and water intake, and rectal temperatures during this time period in order to estimate metabolic changes.

(2) Various types of shock were produced in the following ways: (a) burn: mice were immersed to the level of the iliac crests in water at 70° C for 5

seconds; (b) tourniquet: rubber bands were placed on one or both hindlegs of mice for 1/2 to 2 hours; (c) *E. coli* endotoxin: 0.01 - 0.05 mg *E. coli* lipopolysaccharide was injected intraperitoneally daily into mice; and (d) hyperosmolar: one dose of 3 ml of 12% glucose solution was injected intraperitoneally into mice. Small full-thickness skin grafts of 2.3 cm diameter were placed on the backs of recipient mice. Recipient mice were BALB/c for DBA donor mouse skin or C3H for C57BL skin.

(3) Swelling of tail was measured by a previously published method after burn and tourniquet trauma. Protease inhibitors were injected either into the tail vein immediately post tourniquet or intraperitoneally before and after burn trauma.

Swelling pressure following trauma by tourniquets or burns was measured by insertion of tail into a small tube with an inflatable balloon. Pressure was regulated by a mercury system adjustable to variable pressures. The technique involves maintenance of pressure at different levels with measurement of reduction of swelling produced by external pressure.

Major Findings:

Experiments on the effect of environmental temperature in burned mice demonstrate that (1) 48 hour or shock mortality is significantly lowest in animals maintained at 25° C compared with 4°, 31°, and 37° C; and (2) late mortality is not significantly different in animals kept at 31° or 25°, in spite of the fact that nitrogen metabolism and body temperature is much less disturbed at the higher temperature.

Experiments on the effect of traumatic and non-traumatic shock on skin allograft survival demonstrate that burn or tourniquet trauma significantly prolonged the survival time of first set grafts, regardless of whether the trauma was applied as early as 21 days before grafting or as late as 7 days postgraft. Daily intraperitoneal injections of *E. coli* lipopolysaccharide, a non-traumatic form of shock, significantly prolonged survival time of first set grafts, although not to the same extent as both types of traumatic shock. On the other hand, hyperosmolar shock one day prior to grafting had no effect on survival time of skin grafts. A common denominator that increased survival time of allografts was tissue damage.

The influence of protease inhibitors (phenyl-methyl-sulfonyl fluoride, ovomucoid, and lima bean inhibitor) on the swelling following thermal and tourniquet trauma to the mouse tail was determined. A pronounced inhibition was observed in burn trauma but not in tourniquet, indicating that proteases are involved in the swelling of burn trauma. An apparatus was developed to measure swelling pressure of tissues following application of tourniquet and burn trauma to the rodent tail.

Significance to Bio-medical Research and the Program of the Institute:

The studies on environmental temperatures in burn injury indicate that the large expenditure of calories due to the loss of water by evaporation can be

lessened by keeping the burned animal at 31° C. These findings may be helpful in the clinical management of burned patients. The studies on the effect of various kinds of shock on skin graft survival may lead to the isolation of a natural product resulting from tissue injury that has immunosuppressive activity. Such a product would be useful in the transplantation of organs. In the studies on swelling after trauma, the experiments will afford information on the role of proteases in this mechanism, which is still largely undetermined. In addition, swelling pressure following trauma has not been accurately measured in previous work. The technique developed here will give information on pressure changes following standardized trauma.

Proposed Course:

We plan to (1) study the mechanism of action of biologic amines on receptors sites by *in vitro* techniques; (2) determine what fraction of the purification steps of phytohemagglutinin is responsible for its action on cellular immunity; (3) isolate the natural product produced by trauma which is responsible for prolongation of graft survival; and (4) to continue investigations on the mechanism of swelling after trauma.

Honors and Awards

None

Publications:

Markley, K., and Smallman, E.: Protection against burn, tourniquet, and endotoxin shock by purine compounds. *J. Trauma*, 10, 598-607 (1970).

Markley, K., Smallman, E., and Thornton, S. W.: The effect of diet protein on late burn mortality. *Proc. Soc. Exptl. Biol. Med.*, 135, 94-99 (1970).

Rosenthal, S. M., and Thornton, S.: Influence of temperature on swelling following experimental thermal or tourniquet trauma. *Am. J. Physiol.*, 219, 131-135 (1970).

Serial No. NIAMD-LBP-4
1. Biochemical Pharmacology
2. Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Biology of Complex Carbohydrates

Previous Serial Number: Same

Principal Investigator: Dr. Victor Ginsburg

Other Investigators: Drs. Akira Kobata, Catherine A. Hickey, and Teresa Sawicka

Cooperating Units: None

Man Years

Total:	6.8
Professional:	3.8
Others:	3.0

Project Description:

Objectives:

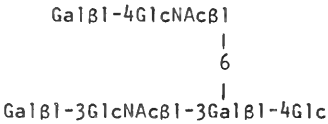
To investigate the role of complex carbohydrates in cell biology.

Methods Employed:

Usual biochemical techniques.

Major Findings:

A new hexasaccharide has been isolated from human milk and its structure determined:



This sugar is especially interesting for two reasons: (1) its branched structure is identical to the structure proposed by Kabat [*cf., Proc. Nat. Acad.*

Sci., 61, 1470 (1968)] for the main chain of blood group determinants and provides proof that his proposal is correct; and (2) the sugar is a potent hapten inhibitor of anti-I sera, suggesting that this structure may be an immunizing antigen in auto-immune hemolytic anemias, many of which result from anti-I cold agglutinins.

Significance to Bio-medical Research and the Program of the Institute:

These studies may contribute to an understanding of cell-cell interactions which are important to biological phenomena such as graft rejection, auto-immune diseases, and carcinogenesis.

Proposed Course:

We will continue to try to elucidate the carbohydrate structures on cell surfaces that are responsible for their specificity.

Honors and Awards

None

Publications:

Ginsburg, V.: Isolation of sugar nucleotides. In Whistler, R. (Ed.): *Methods in Carbohydrate Chemistry*, Volume VI. New York, New York, Academic Press, 1971, in press.

Ginsburg, V., and Kobata, A.: Carbohydrate structures of mammalian cell surfaces. In Rothfield, L. (Ed.): *Membrane Structure and Function*. New York, New York, Academic Press, in press.

Kobata, A.: Heterosaccharide compounds of milk. In Suzuki, S., Matsumura, T., and Yamashina, I. (Eds.): *Polysaccharide Biochemistry*. Tokyo, Japan, Kyoritsu Press, 1969, pp. 1019-1041.

Kobata, A.: Human milk oligosaccharides and blood groups. *Journal of Japanese Biochemical Society*, 42, 829-841 (1970).

Kobata, A., Grollman, E. F., Torain, B., and Ginsburg, A.: Genes, glycosyltransferases and blood types. In Aminoff, D. (Ed.): *Blood and Tissue Antigens*. New York, New York, Academic Press, 1970, pp. 497-506.

Serial No. NIAMD-LBP-5A
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Estimation, Metabolism, and Function of Amines

Previous Serial Number: Same

Principal Investigators: Dr. Herbert Tabor and Dr. Celia White Tabor

Other Investigators: Dr. William H. Bowman and Mrs. Patricia Deal Kellogg

Cooperating Units: None

Man Years

Total:	5.3
Professional:	3.8
Other:	1.5

Project Description:

Objectives:

The importance of the various polyamines is shown by their wide distribution in viruses, bacteria, plant cells, and animal cells. Studies are being conducted to elucidate their metabolism and function.

Methods Employed:

The amines and their derivatives are studied by isotopic labeling and special chromatographic techniques. Standard enzymological techniques have been used to purify further the enzymes that synthesize and degrade spermine, spermidine, and monoamines. Chemical synthetic procedures have been utilized to prepare derivatives for identification of enzymatic products. Uptake of amines was studied by counting the residual radioactivity in washed pellets on Millipore filters. Gel electrophoresis techniques have been utilized to study the ribosome pattern of cells. Amino acid analyses were carried out in an automatic amino acid analyzer. Fluorometric analyses have been utilized.

Major Findings:

The studies on the biosynthesis of spermidine in *Escherichia coli* have included work on the propylamine transferase using a spectrophotometric assay for this enzyme, depending on the use of the spermidine dehydrogenase from

Serratia marcescens, and we have obtained the enzyme about 1,600-fold purified. The enzyme is homogeneous by physical-chemical studies. We have found no requirement for added cofactors.

As an extension of our studies on changes in polyamines of *E. coli* during storage in the cold, we have begun to study the changes of ribosome patterns in the cold which were described by Rich and coworkers. We have continued these studies to determine if any correlation exists with amine acetylation.

We have isolated and studied the structure of the glutathione-spermidine derivative which was first observed by Dubin when he was in our laboratory. We are studying conditions under which this derivative is formed. We are using enzymological and chemical procedures to define its structure. We have found conditions which result in the rapid conversion of spermidine to the glutathionyl derivative, and also in the rapid breakdown of the derivative. An enzyme has been partially purified from *E. coli* extracts which carries out the hydrolysis of the disulfide of glutathionylspermidine. We are now characterizing the product of this reaction.

Significance to Bio-medical Research and the Program of the Institute:

Recent work has indicated that the polyamines may play an important role in permeability of organisms and in the stability of nucleic acids inside cells. Recent studies by Pardee and coworkers on synchronous cultures of *E. coli* suggest that the amines may also play an important role in the division of *E. coli*.

Our findings of changes in the amine content and patterns in *E. coli* after exposure to cold may be only one manifestation of adaptation to an unfavorable environment. Recently Rich and coworkers have shown that ribosomal patterns change under these conditions. It is possible that the amine changes can be correlated with the changes in ribosome patterns.

Our studies on the propylamine transferase enzyme may lead to elucidation of the mechanism of the formation of the C-N bond in bacteria and the role of the high-energy sulfonium ion in this reaction.

Proposed Course:

Further studies on the metabolism and function of the biologically important amines. We hope to learn the normal physiological function of these amines in the cell and to explain such pathological effects as the production of renal damage.

Honors and Awards

Herbert Tabor - Meritorious Service Medal, National Institutes of Health, 1970.

Herbert Tabor appointed as Editor-in-Chief of *The Journal of Biological Chemistry* for a five-year period beginning July 1, 1971.

Publications:

Tabor, C. W., and Kellogg, P. D.: Identification of flavin adeninine dinucleo-

tide and heme in a homogeneous spermidine dehydrogenase from *Serratia marcescens*. *J. Biol. Chem.*, 245, 5424-5433 (1970).

Tabor, C. W., and Tabor, H.: The complete conversion of spermidine to a peptide derivative in *Escherichia coli*. *Biochem. Biophys. Res. Commun.*, 41, 232-238 (1970).

Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*. New York, Academic Press, 1970, Volume 17, Part A, 1,098 pp., and 1971, Part B, 961 pp.

Rechler, M. M., and Tabor, H.: Histidine ammonia-lyase (*Pseudomonas*). In Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*, Volume 17, Part B. New York, Academic Press, 1971, pp. 63-69.

Tabor, C. W.: The determination of NH_3 with the use of glutamic dehydrogenase. In Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*, Volume 17, Part A. New York, Academic Press, 1970, p. 955.

Tabor, C. W., and Kellogg, P. D.: Spermidine dehydrogenase (*Serratia marcescens*). In Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*, Volume 17, Part B. New York, Academic Press, 1971, pp. 746-753.

Tabor, H., and Tabor, C. W.: Enzymatic synthesis of radioactive S-adenosyl-L-methionine. In Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*, Volume 17, Part B. New York, Academic Press, 1971, pp. 393-397.

Tabor, H., and Tabor, C. W.: Glutathionylspermidine synthetase (*Escherichia coli*). In Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*, Volume 17, Part B. New York, Academic Press, 1971, pp. 815-817.

Tabor, H., Tabor, C. W., and DeMeis, L.: Chemical synthesis of N-acetyl-1,4-diaminobutane, N^1-acetylspermidine, and N^8-acetylspermidine. In Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*, Volume 17, Part B. New York, Academic Press, 1971, pp. 829-834.

Wickner, R. B., Tabor, C. W., and Tabor, H.: Adenosylmethionine decarboxylase (*Escherichia coli* W). In Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*, Volume 17, Part B. New York, Academic Press, 1971, pp. 647-651.

Wickner, R. B., and Tabor, H.: N-Formimino-L-glutamate imino-hydrolase (*Pseudomonas sp.*). In Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*, Volume 17, Part B. New York, Academic Press, 1971, pp. 80-84.

Serial No. NIAMD-LBP-5B
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Formiminoglutamate Imino Hydrolase

Previous Serial Number: Same

Principal Investigators: Dr. Reed B. Wickner and Dr. Herbert Tabor

Other Investigators: None

Cooperating Units: None

Man Years

Total:	0.0
Professional:	0.0
Other:	0.0

This project has been discontinued, except for preparation of the material for publication.

Serial No. NIAMD-LBP-6
1. Biochemical Pharmacology
2. Biochemical Regulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Enzymatic Studies of Nucleic Acid Metabolism

Previous Serial Number: Same

Principal Investigator: Dr. Nancy G. Nossal

Other Investigator: Dr. Michael S. Hershfield

Cooperating Units: None

Man Years

Total:	2.5
Professional:	2.0
Others:	0.5

Project Description:

Objectives:

To determine the characteristics and functions of enzymes induced by T_{even} phage which are involved in the synthesis, modification, and degradation of DNA.

Methods Employed:

Standard biochemical techniques were employed for the preparation of polynucleotide substrates and enzymes. Extensive use has been made of amber mutants of T4 bacteriophage which are unable to make specific early enzymes in nonpermissive hosts. Procedures have been developed for the large scale preparation of these amber mutants and for the production of kilogram quantities of infected bacterial cells.

Major Findings:

The DNA polymerase induced by T4 bacteriophage has both polymerase and exonuclease activities. We had shown previously that after infection of a nonpermissive host with *am* B22, an amber mutant in the gene for the T4 DNA polymerase, there is no phage DNA polymerase activity, but there is an exonuclease activity similar to that in the wild type enzyme. It seemed

probable that the mutant nuclease was due to a polypeptide fragment of the wild type enzyme extending from the N terminal end of the protein to the point corresponding to the *am* B22 mutation, which is 80% of the way from one end of the gene. We have now demonstrated that the mutant nuclease is an amber fragment of the wild type enzyme by showing that both proteins, which have been purified to apparent homogeneity, are single polypeptide chains and that the molecular weight of the mutant by gel electrophoresis in sodium dodecyl sulfate and mercaptoethanol or by high speed equilibrium sedimentation is about 80% of that of the wild type protein. Furthermore, the mutant was shown to contain most but not all of the tryptic peptides of the wild type enzyme by cochromatography of the tryptic peptides of the two proteins after differential labeling with radioactive iodoacetic acid.

Both the mutant and wild type enzymes have been shown to degrade denatured DNA from the 3' chain terminus randomly rather than processively (one chain at a time) since they selectively hydrolyze labeled nucleotide at the 3' terminus during the initial stages of the digestion. Although the degradation is random, DNA chains of intermediate length are not found after partial digestion of DNA by either enzyme since the smaller chains are degraded at a much faster rate than the initial substrate. There is no evidence for a 5' exonuclease activity, like that in the *E. coli* DNA polymerase, in either the wild type or mutant phage proteins. The mutant enzyme differs from the wild type in its markedly decreased affinity for DNA, oligonucleotides, and deoxynucleoside triphosphates. The *am* B22 enzyme is unable to synthesize DNA or to carry out the terminal addition of a single nucleotide even when the template and substrates are present at high concentrations.

In studies designed to elucidate the role of the exonuclease activity of the wild type T4 polymerase during DNA synthesis, we have confirmed the observation of Goulian, Lucas, and Kornberg that the nuclease activity is strongly inhibited in the presence of deoxynucleoside triphosphates, and that label incorporated into DNA *in vitro* is not degraded after net synthesis has ceased. While these results suggested that the nuclease did not function during synthesis, we have found by following the fate of the nucleoside triphosphates that they are not only incorporated into DNA but are also converted into free nucleoside monophosphates. The conversion of triphosphate into monophosphate continues after incorporation has ceased and shares the following requirements with DNA synthesis. It is specific for deoxynucleotides which can be incorporated, requires DNA as a template and a free 3-hydroxyl terminus to serve as primer, and is inhibited by 3' phosphate termini. Thus dATP is converted to dAMP if the homopolymer pair $(dA)_n \cdot (dT)_n$ is used as template-primer, but not if either homopolymer is omitted or if $(dI)_n \cdot (dC)_n$ is used instead. Our current interpretation is that this conversion of deoxynucleoside triphosphate into monophosphate is due to hydrolysis at or very close to the end of the newly synthesized DNA by the exonuclease activity of the T4 DNA polymerase.

Significance to Bio-medical Research and the Program of the Institute:

Knowledge of enzymes controlling DNA synthesis and degradation is important to an understanding of the control of DNA replication and transcription

in both normal and viral infected cells.

Proposed Course:

The role of the exonuclease in DNA synthesis by the wild type enzyme and the requirements of the wild type enzyme for the binding of template and substrates and for phosphodiester bond formation will be studied by a detailed comparison of the structural and enzymatic properties of the wild type enzyme with that of amber and temperature sensitive mutants in this gene. The function of the T4 DNA polymerase in the infection process will continue to be investigated.

Honors and Awards

None

Publications:

None

Serial No. NIAMD-LBP-7
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Protein Synthesis in *Escherichia coli*

Previous Serial Number: Same

Principal Investigator: Dr. Anthony V. Furano

Other Investigator: Dr. Maureen I. Harris

Cooperating Units: None

Man Years

Total:	2.1
Professional:	2.0
Others:	0.1

Project Description:

Objectives:

To study the mechanism and control of protein synthesis *in vivo* and *in vitro*.

Methods Employed:

In vivo. We have been able to isolate and begin to study a mutant of *E. coli* which cannot synthesize protein at 42°.

In vitro. We have been investigating the functional capacities of ribosomes that have been modified either by treatment with a selective chemical reagent or by genetic alteration. The modified ribosomes are tested for their ability to participate in the various stages of protein synthesis *in vitro*: *i.e.*, chain initiation, chain elongation, chain termination. We are also studying the soluble enzymes that catalyze in some way polypeptide chain elongation.

Major Findings:

(1) Studies *in vivo*. We have continued our work on the temperature sensitive mutant of *E. coli* which was described in the previous Annual Report.

We have established that inability of the cell to accumulate protein at 42° is due to a decrease in synthetic ability and not due to a massive increase in protein degradation. This result, coupled with our earlier data on the stability of polysomes at 42° and the persistence (for a short time at least) of RNA and DNA synthesis at 42°, suggests that the lesion in this mutant may reside in a heretofore unrecognized part of the cell's protein synthesizing apparatus.

(2) Studies *in vitro*. We have completed our studies on the effect of oxidized glutathione (GSSG) on ribosomes and have made the following observations: (1) Prolonged (24 hour) anaerobic incubation at 37° of ribosomes in the *absence* of GSSG leads to extensive degradation of all of the ribosomal RNA to pieces with a sedimentation in sucrose gradients of about 5S; (2) Such ribosomes are essentially fully active when assayed for poly U directed polyphenylalanine synthesis; and (3) If the above incubation includes GSSG (0.05 M), then all of the 70S ribosomes are dissociated into 30S and 50S subunits *and* the RNA is degraded in a manner different from ribosomes incubated in the absence of GSSG. We showed that this different pattern is not due to the dissociation *per se* but due to the fact that the GS- moiety of GSSG is bound to the ribosomal protein of both the 30S and 50S subunits. These observations suggest: (a) intact ribosomal RNA is not required for ribosomal function; (b) there is some structural periodicity to the ribosome so as to allow the nucleolytic production of 5S pieces; and (c) the GSSG via its interaction with ribosomal protein has altered the structure of the ribosome as reflected in a different manner of RNA degration.

These studies also have confirmed and extended earlier reports from this laboratory (and that of Traut and Haenni) that ribosomal SH's are involved in subunit association into 70S ribosomes, the binding of phe tRNA by the 30S subunit, but not the binding of poly U by the 30S subunit.

Significance to Bio-medical Research and the Program of the Institute:

Knowledge on the mechanism of protein synthesis is essential to understanding cellular metabolism and growth.

Proposed Course:

This work is currently in progress and will be continued and extended.

Honors and Awards

None

Publications:

None

Serial No. NIAMD-LBP-8
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Role of Transfer RNA in Animal Virus Infection

Previous Serial Number: NIAMD-LBP-8b

Principal Investigator: Dr. Michael Klagsbrun

Other Investigators: None

Cooperating Units: None

Man Years

Total:	1.1
Professional:	1.0
Other:	0.1

Project Description:

Objectives:

To determine whether infection with DNA viruses such as vaccinia, Herpes, and SV40 cause any changes in the methylation pattern of cellular transfer RNA.

Methods Employed:

Standard tissue culture and biochemical techniques such as column and thin layer chromatography, sucrose gradients, and gel electrophoresis.

Major Findings:

An 8 hour infection of HeLa cells by vaccinia virus leads to a change in the elution pattern on B-D cellulose of labeled cellular tRNA. A comparison of uridine- and methyl-labeled tRNA suggests that the tRNA synthesized in infected cells is about twice as methylated as in uninfected cells.

Thin layer chromatography of acid hydrolyzed methyl-labeled tRNA indicates that no methylated bases are found in infected cell tRNA that are not also found in uninfected cell tRNA. The possibility remains that new sequences of methylated tRNA exist in infected cells that do not exist in uninfected cells.

Significance to Bio-medical Research and the Program of the Institute:

It has been claimed that cytoplasmic extracts of tumor cells with a viral (SV40, adeno 12) origin, have much higher methylase activities than do extracts of normal cell controls. As a result possible differences might exist in the structure of the tRNA of tumor and normal cells, and these differences could play a role in the regulation of protein synthesis in these types of cells.

At present, no studies have shown whether differences in methylation occur in methyl-labeled tRNA extracted from normal and infected cells. My studies with vaccinia, SV40, and Herpes (all of which can cause transformation, are an attempt to shed light on this problem.

Proposed Course:

Comparisons will be made in the methylated base patterns and methylated oligonucleotide sequences of methyl-labeled tRNA extracted from infected, transformed, and control cells. The systems being used are HeLa and vaccinia infected HeLa, Hep-2 and Herpes infected Hep-2, monkey and SV40 infected monkey, and 3T3 and SV3T3 transformed cells.

Honor and Awards

None

Publications:

Klagsbrun, M.: Changes in the methylation of transfer RNA in vaccinia infected HeLa cells. *Virology*, in press.

Serial No. NIAMD-LBP-9
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Chemical and Physiological Properties of the Surface of *Escherichia coli*

Previous Serial Number: Same

Principal Investigator: Dr. Loretta Leive

Other Investigators: Dr. David C. Morrison and Dr. David A. Lawrence

Cooperating Units: None

Man Years

Total:	2.5
Professional:	2.2
Other:	0.3

Project Description:

Objectives:

To study the chemical and physiological properties of the cell surface of *E. coli*.

Methods Employed:

Usual biochemical methods, including isolation and purification of cell surface components by extraction, ultracentrifugation, chromatography, and selective enzymatic degradation, determination of the size and shape of these fractions by biophysical measurements of density, viscosity, and ultracentrifugal properties, identification and quantitation of sugars by colorimetric and enzymatic procedures, and use of labeled compounds to specifically label cell fractions *in vivo*.

Major Findings:

The lipopolysaccharide (LPS) of *E. coli* is believed to be located primarily or entirely in the outer cell wall layer. Our previous findings indicated that exposure of whole cells of *E. coli* to EDTA causes release of half of this LPS. Current experiments have provided information on the relationship of this releasable LPS to the remainder.

To determine the location of the releasable LPS relative to the remainder, the adsorption of ε15 phage to *Salmonella anatum* was measured, since this adsorption is known to be dependent on LPS. The results show that at low multiplicities the rate and extent of adsorption is equal in EDTA-treated and control bacteria, suggesting that there is no barrier to the accessibility of phage to the remaining LPS. At high multiplicities, only half as many phage attach to EDTA-treated as to control bacteriia. These results indicate that the EDTA treatment does not expose a new, previous inaccessible LPS layer, but rather that the two LPS fractions are equivalently accessible to phage. It is thus possible to postulate that they are equally external in their location.

Experiments were also performed in an attempt to determine why only half of the LPS is released by EDTA. The results show that although under normal growth conditions approximately half of the total LPS of *E. coli* is releasable by EDTA, if the concentration of divalent cations present during growth is greatly increased, the fraction of LPS subsequently releasable by EDTA can also be increased. These results are consistent with previous isotopic evidence that the releasable LPS is in metabolic equilibrium with the remainder. One may postulate the following equation: $LPS\text{-}X^{+} \rightleftarrows LPS\text{-}Me^{++}$, where X is an unknown cationic grouping that cannot be chelated by EDTA.

In other experiments, a new method for extracting and purifying LPS has been devised, employing treatment of cells with aqueous butanol-1, followed by pronase digestion and ultrafiltration. The resultant LPS is at least 95% pure and shows properties different from that of LPS isolated by conventional means. In particular, the density of material prepared by this method is less than that of LPS prepared by the standard (phenol) extraction, which suggests a higher lipid content. Nevertheless, the usual methods for assaying the lipid moiety of LPS (lipid A) reveal only 10-15% of this fraction in material isolated by the new method as in material isolated by phenol extraction. The reasons for these contradictory results are under investigation. Since the butanol-extracted LPS can be modified by the action of phenol to resemble phenol-extracted LPS in density, it is possible that the butanol extraction retains the LPS in a more nearly native configuration.

Significance to Bio-medical Research and the Program of the Institute:

Study of the organization of the outer layer of the cell surface will contribute to understanding its structure and its interaction with the environment. In addition, since the above mentioned EDTA treatment causes an increase in passive permeability, these studies will elucidate the relationship of the cell surface structure to the normal cellular permeability barrier. When this barrier is altered by EDTA treatment, drugs normally excluded can enter, so understanding the nature of the changes caused by this treatment may have implications for drug therapy.

Proposed Course:

The chemical and physiological effects of shifting the equilibrium postulated above will be measured. Attempts will also be made to isolate *E. coli*

cell surfaces and to shift the LPS equilibrium by adding high concentrations of divalent cations. It may then be possible to dissociate all of the LPS, and perhaps the entire outer cell wall, by adding EDTA. In other experiments the differences between LPS isolated by the new butanol method and LPS isolated by more conventional methods will be explored.

Honors and Awards

None

Publications:

Voll, M. J., and Leive, L.: Actinomycin resistance and actinomycin excretion in a mutant of *Escherichia coli*. *J. Bacteriol.*, 102, 600-602 (1970).

Serial No. NIAMD-LBP-10
1. Biochemical Pharmacology
2. Biochemical Regulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Chemistry and Mechanism of Pyridoxal Phosphate Enzymes

Previous Serial Number: Same

Principal Investigator: Dr. Edith Wilson Miles

Other Investigators: Drs. Jerry B. Gin and Hidehiko Kumagai

Cooperating Units: Drs. I. P. Crawford and Richard Maurer of the Scripps Clinic and Research Foundation, La Jolla, California

Man Years

Total:	2.0
Professional:	1.8
Other:	0.2

Project Description:

Objectives:

To gain further understanding of features of enzymes which contribute to their conformation, subunit interaction, and mechanism of action. Pyridoxal phosphate enzymes have been chosen for study because they are important in amino acid metabolism and because pyridoxal phosphate has optical and chemical properties which make it a useful probe of the active site of the enzymes to which it is bound.

Methods Employed:

Chemical modification studies are carried out on highly purified pyridoxal phosphate enzymes (L-tryptophan synthetase and L-aspartate decarboxylase) to determine the presence and role of specific functional groups at the active sites and subunit combining sites of the enzymes. Amino acid analyses and peptide isolation procedures are carried out to identify the modified residues. Optical studies are carried out on the native and modified enzymes to determine changes in the environment and reactive intermediates of the chromophoric coenzyme, pyridoxal phosphate, and aromatic amino acids. Kinetic studies are conducted to determine the effects of specific modification on subunit interactions, cofactor and substrate binding, and reaction specificities.

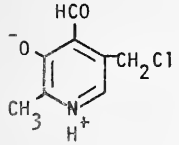
Substrate and cofactor analogs are synthesized which are designed to modify the active site of enzymes. Reactions with substrate analogs are studied to more fully define reaction mechanism and specificity.

Major Findings:

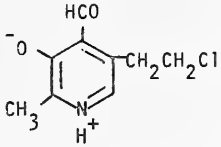
Tryptophan synthetase of *Escherichia coli* has been shown by other workers to be an interacting system of two different protein subunits, the A and B proteins, which carry out a variety of reactions subject to numerous controls. In order to determine factors involved in the interaction of these two proteins, chemical modification reactions of the B protein are being carried out in the presence and absence of the A protein, substrates, and cofactor, and properties of the modified proteins are being studied.

Chemical modification studies of the sulfhydryl residues of the B protein of *Escherichia coli* tryptophan synthetase have been completed and have increased our understanding of the active site and of the subunit interaction site of this enzyme. Comparative studies of the sulfhydryls of the B protein of *Pseudomonas putida* tryptophan synthetase have been conducted. Peptides containing sulfhydryl residues specifically labeled with ^{14}C-N-ethyl maleimide are currently being isolated from tryptic digests of the B proteins of the two bacterial species and appear to be different. These studies are important in understanding differences which have evolved in the active site regions of the two B proteins, particularly since another active site peptide has been found to be very similar in the B proteins from the two organisms.

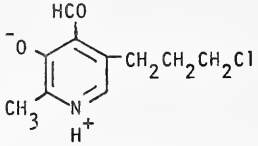
Three analogs of pyridoxal phosphate, the cofactor for tryptophan synthetase and many other enzymes essential in amino acid metabolism, have been synthesized and studied as possible active site directed affinity labels and inhibitors. These analogs (compounds I-III) all react irreversibly and covalently with the apo B protein of tryptophan synthetase.



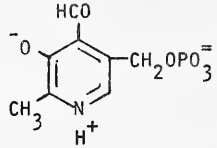
I α^5-pyridoxal chloride



II α^5-pyridoxal methylchloride



III α^5-pyridoxal ethylchloride



IV α^5-pyridoxal phosphate

α^5-Pyridoxal methylchloride (II) appears to be the most specific of the analogs and is being studied further. It reacts almost stoichiometrically with the apo B protein at a rate which is greatly decreased by the natural cofactor, pyridoxal phosphate (IV). A second covalent attachment of the analog to the active site of the enzyme can be introduced by $NaBH_4$ reduction of the Schiff base which is formed between the carbonyl group of the analog and an ε-amino group of lysine in the protein. Thus the new analog can be used as a cross

linking reagent between two residues in the cofactor binding site. The product of alkylation by the analog is currently being isolated from acid hydrolysates of the modified protein and will be identified by comparison with standards which are being prepared by synthesizing various amino acid derivatives of α^5-pyridoxal methylchloride. Work on isolating the labeled active site peptide from tryptic digests of the B protein from both *Ps. putida* and *E. coli* is underway. The analog should prove to be useful in comparative studies of the pyridoxal phosphate binding site in various pyridoxal phosphate enzymes.

Studies of the substrate and reaction specificity of tryptophan synthetase have been carried out in order to extend our understanding of the reaction mechanism of the enzyme and to compare it with the closely related degradative enzyme, tryptophanase. Several new reactions of the B protein of tryptophan synthetase with β-substituted amino acid substrates have been found: β-elimination reactions with S-methyl-L-cysteine and O-methyl-L-serine to produce pyruvate (reaction 1) and β-replacement reactions with the same substrates plus indole (R'H) to produce L-tryptophan (reaction 2) where RH = CH_3SH or CH_3OH.

$$RCH_2CHNH_2COOH + H_2O \rightleftharpoons CH_3COCOOH + RH + NH_3 \quad (1)$$

$$RCH_2CHNH_2COOH + R'H \rightleftharpoons R'CH_2CHNH_2COOH + RH \quad (2)$$

The primary differences between the two enzymes are (1) the inability of tryptophan synthetase to catalyze reaction 1 where RH = indole and (2) the inability of tryptophanase to catalyze reaction 2 where R'H is CH_3SH or CH_3OH. These findings increase our understanding of the mechanistic controls over the two enzymes.

Significance to Bio-medical Research and the Program of the Institute:

These studies are clarifying the active site chemistry and mechanism of pyridoxal phosphate enzymes which are important in amino acid metabolism.

Proposed Course:

These studies will be continued and extended with long range goals of determining chemical and physical features of pyridoxal phosphate enzymes required for specificity, catalytic activity, maintenance of conformation, and protein-protein interaction.

Honors and Awards

None

Publications:

Miles, E. W.: The B protein of *Escherichia coli* tryptophan synthetase. I. Effects of sulfhydryl modification on enzymatic activities and subunit interaction. *J. Biol. Chem.*, 245, 6016-6025 (1970).

Miles, E. W., and Sparrow, L. G.: L-Aspartate-β-decarboxylase (*Achromobacter*). In Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*, Volume 17, Part A. New York, Academic Press, 1970, pp. 689-693.

Tate, S. S., Novogrodsky, A., Soda, K., Miles, E. W., and Meister, A.: L-Aspartate-β-decarboxylase (*Alcaligenes faecalis*). In Tabor, H., and Tabor, C. W. (Eds.): *Methods in Enzymology*, Volume 17, Part A. New York, Academic Press, 1970, pp. 681-688.

Serial No. NIAMD-LBP-11
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Molecular Chemistry of Enzyme Specificity

Previous Serial Number: Same

Principal Investigator: Dr. Leonard D. Kohn

Other Investigator: Dr. John M. Utting

Cooperating Units: (1) Mr. George Poy, Arthritis and Rheumatism Branch, NIAMD, on Amino Acid Analyses
(2) Dr. Roger J. Winand, Hopital Universitaire de Baviere, Institut de Medecine, Liege, Belgique
(3) Dr. Ivan G. Darvey, Physical Sciences Laboratory, Division of Computer Research and Technology
(4) Mr. Richard Shrager, Physical Sciences Laboratory, Division of Computer Research and Technology

Man Years

Total:	2.1
Professional:	2.0
Others:	0.1

Project Description:

Objectives:

Analyses of the molecular bases for enzyme activity and specificity and the mechanisms by which such factors are regulated or determined. Studies of abnormal hormonal factors, their production, and their enzymatic expression.

Methods Employed:

Procedures common to the purification, assay, and chemical modification of enzymes have been coupled with methods evaluating the size, shape, composition, and sequence of macromolecules.

Major Findings:

Structure function studies of related reductases have demonstrated that the catalytic mechanism is independent of their size and subunit structure,

that a common active site peptide exists and is related to the catalytic peptide of glyceraldehyde phosphate dehydrogenase, and that specificity and regulation are associated with secondary structure and configurational changes. Subunit reassociation has been demonstrated for several reductases. Reassociation is significantly influenced by pH, temperature, glycerol concentration, salt concentration, and buffer composition. Subunit conformation is important for optimal association; the formation of interchain disulfide bonds is not important for association but rather for the stabilization of the associated state. The kinetics of association approach theoretical in models where interchain disulfide bonds are absent and an inverse concentration dependence is seen where wrong disulfide pairings can yield nonproductive complexes. Association is highly specific since similarly sized subunits from hydroxypyruvate reductases of different origins reassociate poorly or not at all. Exogenous proteins, including those in crude cell extracts, do not hinder association but can improve it by modifying the rate of the reverse reaction, dissociation.

Studies on the relationship of thyrotropin (TSH) to exophthalmos-producing substance (EPS) have confirmed that the determinants for both activities can reside on the same bovine pituitary glycoprotein. Partial pepsin digestion destroys the TSH activity of crude and purified extracts whereas their EPS action is preserved. A glycopeptide isolated from these pepsin digests has been found to have EPS activity but no significant TSH action; its molecular weight on SDS gels is approximately 20,000. After preincubation with 0.1 M mercaptoethanol as well as SDS, two fragments are visualized on SDS gels, one having a molecular weight of 14,000, the other of 6,000. Preliminary data suggest that the glycopeptide is composed of both subunits of TSH, the B subunit being relatively intact and the α partially destroyed. The EPS glycopeptide is active in stimulating mucopolysaccharide synthesis in the mouse and guinea pig harderian gland; sulfate activation is one of the first events in this process.

Human TSH is similarly affected by partial pepsin digestion; tumor TSH unmasks a significant EPS activity during the digestion process. Both subunits of bovine TSH appear to have EPS as well as TSH activity, and the ratio of EPS to TSH action is higher than in the native molecule. Both subunits are less active than the EPS glycopeptide isolated from pepsin digests.

Significance to Bio-medical Research and the Program of the Institute:

Analyses of the molecular bases for activity and specificity and the mechanisms by which these determinants are influenced offers a further understanding of the nature of metabolic and endocrine defects. The possibility that unregulated biosynthetic or degradative processes may convert TSH into EPS active material has been established. This work is pursued with the hope that such understanding will aid in attacks on related disease processes.

Proposed Course:

Structure-function analyses of the reductase model system are being pursued. The localization of the active and regulatory sites on each subunit will be sought and the comparative structures of these units will be compared.

Correlation with the kinetics of modified proteins and substrates will be attempted. Comparison of membrane bound and free enzymes will be made. Similar studies of the TSH-exopthalmic factor relationship will be continued in an attempt to ascertain the structural basis for the two activities and to examine the mechanism by which EPS activity is generated *in vitro* and *in vivo*. An immuno-assay will be developed for EPS using the uniquely active EPS glycopeptide.

Honors and Awards

None

Publications:

Kohn, L. D.: Renaturation of spinach leaf glyoxylic acid reductase. *J. Biol. Chem.*, 245, 3850-3858 (1970).

Kohn, L. D., Hurlbert, R. G., and Jakoby, W. B.: L-(+)-Tartrat und meso-tartrat. In Bergemeyer, H. U. (Ed.): *Methoden der Enzymatischen Analyse*, 2nd Edition. Weinheim (Germany), Verlag-Chemie, 1970, pp. 1360-1366.

Kohn, L. D., and Warren, W. A.: The kinetic properties of spinach leaf glyoxylic acid reductase. *J. Biol. Chem.*, 245, 3831-3839 (1970).

Kohn, L. D., Warren, W. A., and Carroll, W. R.: The structural properties of spinach leaf glyoxylic acid reductase. *J. Biol. Chem.*, 245, 3821-3830 (1970).

Warren, W. A., and Kohn, L. D.: Sulfhydryl studies of spinach leaf glyoxylic acid reductase. *J. Biol. Chem.*, 245, 3840-3849 (1970).

Serial No. NIAMD-LBP-12
1. Biochemical Pharmacology
2. Biochemical Regulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Role of Subunits Interactions in Enzyme Chemistry

Previous Serial Number: Same

Principal Investigator: Dr. Claude B. Klee

Other Investigators: None

Cooperating Units: Dr. Werner Klee, Laboratory of General and Comparative Biochemistry, National Institute of Mental Health
Dr. Jules A. Gladner, Laboratory of Biophysical Chemistry, National Institute of Arthritis and Metabolic Diseases

Man Years

Total:	1.2
Professional:	1.0
Others:	0.2

Project Description:

Objectives:

Two enzymatic systems, histidine ammonia-lyase and lactose synthetase, are used to study the possible regulatory functions of protein-protein interactions.

Methods Employed:

The project involves the study of properties of proteins, purified by the usual techniques of protein isolation, including many types of chromatography. The enzymes are being studied by examining their optical, hydrodynamic, and kinetic properties as well as by suitable chemical measurements and modifications.

Major Findings:

Histidine ammonia-lyase was previously shown to be composed of 4 subunits which are identical in size. In collaboration with Dr. Gladner, we have now shown that each subunit contains one of the 4 reactive cysteines which are necessary for maximum enzymatic activity. Treatment of the thiol reduced

enzyme with ^{14}C iodoacetate was followed by a decrease in specific activity and carboxymethylation of 3.6 - 3.8 cysteinyl residues per mole of enzyme. The ^{14}C labeled enzyme still contains 12 buried cysteines which were carboxymethylated in 8 M urea - mercaptoethanol with ^{12}C reagent. A tryptic digest of this fully carboxymethylated enzyme revealed a single radioactive peptide on gel filtration on a Sephadex G50 (superfine) column and on a conventional peptide map. The radioactive peptide was shown to be composed of 18 amino acid residues and it contains 1 carboxymethyl cysteine residue. These data suggest that each of the 4 subunits of the enzyme is not only of the same size but also contains an identical peptide sequence containing the cysteinyl residue necessary for enzymatic activity. Although the 4 cysteinyl residues are located on 4 identical peptides and are all equally necessary for enzymatic activity, preliminary evidence indicates that they are not equally reactive suggesting that their local environment may differ somewhat. These differences could be the result of intersubunit interactions in the intact enzyme.

A long standing problem which we have taken up in the past year is that of the involvement of metal ions in the reaction catalyzed by histidine ammonia-lyase. The purified enzyme was shown to be free of metal ions and is dependent on the addition of metal (Mn^{++}, Fe^{++}, Zn^{++}, or Cd^{++}) for maximum enzymatic activity when assayed under the appropriate conditions. The well known stimulation of the enzyme by treatment with thiols was shown to be due to an increased affinity of the enzyme for the metal cofactor as measured by enzymatic activity as a function of metal concentration and also by equilibrium dialysis. The divalent metals, Zn^{++}, Cd^{++}, and Mn^{++}, were shown to interact with the 4 free -SH groups present in the reduced enzyme, since they render the protein inactive towards Ellman's reagent. Although Mn^{++} binding to the sulfhydryl groups requires the simultaneous presence of histidine, efficient binding of Cd^{++} and Zn^{++} takes place even in the absence of substrate. However, L-histidine affects the nature of the binding of Cd^{++} and Zn^{++} as well, since these metals are readily removed by EDTA in the absence but not in the presence of L-histidine. Kinetic measurements also indicated that histidine facilitates the binding of the metal to the enzyme. Our working hypothesis is that the metal interacts with the imidazole ring of the substrate and may labilize the proton on the β carbon which is released on urocanic acid formation.

In collaboration with Dr. Werner Klee we have pursued our studies on lactose synthetase. Binding of native α lactalbumin to A protein was demonstrated by following the sedimentation of a band of A protein through solutions of α lactalbumin in the analytical ultracentrifuge using the photoelectric scanner and band forming cells as described by Vinograd and his colleagues. Complex formation was found to be dependent on the presence of N-acetyl glucosamine. In other experiments, using nitrated α lactalbumin to allow visualization at 430 mμ, it was found that complex formation could also be demonstrated in the presence of UDP-galactose and Mn^{++}. By these techniques purified A protein was shown to form a 1 : 1 complex with α lactalbumin in the presence of one of the other substrates. Thus, lactose synthetase can be considered to be an enzyme composed of 2 different subunits, a catalytic subunit and a regulatory subunit. In contrast to most enzymes of this type the complex of the 2 subunits is a transient one, since the catalytic subunit is usually present as such in the membrane and functions as a galactosyl transferase with a different acceptor.

Significance to Biomedical Research and the Program of the Institute:

These two oligomeric systems, histidine ammonia-lyase composed of 4 identical subunits and lactose synthetase, a complex of 2 different proteins, are being studied to elucidate the role of protein-protein interactions in the mechanism of their catalytic function. It is expected that they will complement one another in our effort to understand the physiological significance of macromolecular interactions.

Proposed Course:

Future work with histidase will be aimed at clarifying the role of the metal cofactor in the reaction catalyzed by the enzyme. It will include the study of the amino acids residues at the active site of the enzyme and, more particularly, the dehydroalanine residue previously described (Wickner, R. B., *J. Biol. Chem.*, 244, 6550, 1969). Interactions between these different groups will be studied by UV absorption and circular dichroism. Finally, we hope to study the effect of subunit interactions on the enzymatic activity.

The studies with lactose synthetase will continue to focus on the interactions of the A protein with α lactalbumin and the substrates.

Honors and Awards

None

Publications:

Klee, C. B.: Reversible polymerization of histidine ammonia-lyase. The role of sulfhydryl groups in the activity and polymeric state of the enzyme. *J. Biol. Chem.*, 245, 3143-3152 (1970).

Klee, W. A., and Klee, C. B.: The role of α-lactalbumin in lactose synthetase. *Biochem. Biophys. Res. Commun.*, 39, 833-841 (1970).

Serial No. NIAMD-LBP-13A
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Biological Functions of R Factors Isolated into *Escherichia coli* Minicells

Previous Serial Number: Same

Principal Investigator: Dr. Stuart B. Levy

Other Investigator: Paula Norman, Normal Volunteer, Clinical Center

Cooperating Units: None

Man Years

Total:	0.0
Professional:	0.0
Other:	0.0

This project has been discontinued.

Publications:

Levy, S. B.: Resistance of minicells to penicillin lysis: a method of obtaining large quantities of purified minicells. *J. Bacteriol.*, 103, 836-839 (1970).

Levy, S. B., and Norman, P.: Segregation of transferable R factors into *Escherichia coli* minicells. *Nature*, 227, 606-607 (1970).

Levy, S. B.: Physical and functional characteristics of R factor DNA segregated into *Escherichia coli* minicells. Submitted for publication in *J. Bacteriol.*, 1971.

Serial No. NIAMD-LBP-13B
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Endotoxin Induction of Amyloidosis: Localization and Effect of Endotoxin on Subcellular Fractions

Previous Serial Number: Same

Principal Investigator: Dr. Stuart B. Levy

Other Investigators: None

Cooperating Units: (1) Arthritis and Rheumatism Branch, NIAMD; Laboratory of Viral Diseases and Laboratory of Clinical Investigation, NIAID; and Ophthalmology Branch, NINDS.
(2) Dr. A. Azzi, University of Padua, Padua, Italy.

Man Years

Total:	0.0
Professional:	0.0
Other:	0.0

This project has been discontinued.

Publications:

Willerson, J. T., Trelstad, R. L., Pincus, T., Levy, S. B., and Wolff, S. M.: Subcellular localization of *Salmonella enteritidis* endotoxin in liver and spleen of mice and rats. *Infection and Immunity*, 1, 440-445 (1970).

This year has seen the 220 megahertz super-conducting NMR spectrometer put into use. This instrument, jointly operated by LPB and DCRT, has already proved its usefulness in the solution of a variety of organic structural problems and in the detailed study of ribonuclease conformation. It is anticipated that its versatility and power will be increased still further when it is employed for study of ^{13}C NMR, with its supplementary control computer, now being programmed, and with the 55 MHz pulse apparatus being constructed by the Instrument Section, DRS. 1970 has also seen the start of on-line operation of our Honeywell building computer. At the moment this instrument is servicing our spectropolarimeter and UV, IR and Raman spectrometers, and Mr. Jennings is devoting nearly full time to interfacing and software problems.

The past year has been a proud one for LPB. Dr. Richard Podolsky has been elected President of the Society of General Physiologists. Dr. Podolsky also arranged the 1970 annual symposium of the Society and is editing the resulting volume Contractility of Muscle Cells and Related Processes. Dr. Edwin Becker won the Physical Sciences Award of the Washington Academy of Sciences, and his second book, Pulse and Fourier Transform NMR, is in press. Dr. Makio Murayama's basic work on the physical chemistry of sickle cell hemoglobin has borne fruit in the development of a clinical test for sickle cell anemia, titled the "Murayama test" by its developer, Dr. Robert Nalbandian.

Molecular Structure and Behavior

Spectroscopic techniques provide one of the most useful methods for elucidating molecular structure and studying interactions within and between molecules. In the area of electronic spectra and optical activity, Charney and Tsai showed that the optical activity of the α-diketone camphorquinone arises from the dissymmetry of the diketone chromophore and that the highest filled molecular orbital has a symmetry different from that believed since the 1940's. McDiarmid, too, has been concerned with the assignment of electronic states. In isobutene the 1795 A absorption band was assigned to a $\sigma \leftrightarrow \pi$ transition, and the electronic structure of the molecule in the excited state was shown to be significantly altered from that in the ground state.

Milstien and Charney found that the electro-dichroism of poly-adenylic acid depends on ionic strength, with the results indicating that the polymer rod stiffens as ionic strength decreases.

Eaton and Lewis have used polarized electronic spectra from single crystals to learn more about the direction and nature of the transition moments; for uracil the results are in accord with theoretical predictions, but for cytosine the experimental data contradict the previously accepted theory. Similar studies in crystals of hemoglobin derivatives (Eaton and Makinen) show that the spectra depend markedly on the ligands, a result that was unanticipated in view of Perutz's low resolution x-ray studies that had indicated similar conformations for all derivatives.

Related information on other heme derivatives comes from Kon's study by ESR and NMR of the complex between ferricytochrome c and 3-methylsalicylate. The salicylate is found to be bound near the heme iron, resulting in a strong axial distortion of the field around the iron. Kon and Hirasawa observed an unusual type of Cu^{++} -histidine complex, in which the unpaired electron must be assigned to the axial, rather than the in-plane, d orbital of the copper.

Levin and his co-workers are using infrared and Raman spectroscopy, together with appropriate theoretical calculations, to investigate the dynamics and force fields in a number of small molecules. Low temperatures Raman spectra of isobutene and its deuterated isomers permitted the assignment of the fundamental vibrational modes and the determination of the barrier to internal rotation of the two methyl groups (Levin and Harris). Infrared intensities, determined experimentally and calculated quantum mechanically, can provide further information on intramolecular forces, as demonstrated in the studies of PF_3 by Levin and Adams and of SF_4 by Levin and Berney.

Shoup, et al., have investigated the mechanisms of relaxation of ^{13}C in various molecules and have found that in many cases the spin-spin and spin-lattice relaxation times are not equal, as had previously been widely thought. An understanding of relaxation processes is fundamental to the design of optimum experiments for studying the weak ^{13}C NMR signals found in complex molecules. Yeh and Becker have found substantial differences in the widths of the ^{23}Na NMR lines from intracellular and interstitial sodium ions in frog muscle.

Ziffer and Weiss found that several heteroannular cisoid dienes give optical activity opposite in sign to that predicted by the diene rule, but analysis of the results by a more sophisticated treatment shows how they can be reconciled. Ziffer has also studied circular dichroism bands induced in a series of substituted benzophenones by an optically active solvent and in addition has determined the stereochemistry of several recently synthesized cyclohexanediols.

Weiss, Edwards and Weisgraber have studied the structures, biosyntheses, and laboratory syntheses of a number of pigments originating in plants, molds and insects. They developed a surprisingly simple method for synthesizing a perylene-quinone related to the pigment elsinochrome-A and have confirmed a unique biosynthetic pathway for synthesis of plant perinaphthenone pigments in a species of Lacnanthes.

Adams, Sharpless and Jennings continued their investigation of the nature of the visual pigment, rhodopsin. A model system of a phospholipid complexed with 11-cis-retinaldehyde, which displays spectral behavior closely akin to that of rhodopsin, has been studied in detail, both experimentally and by molecular orbital methods. The kinetics followed by this model system in its transformations when exposed to light are also being followed spectrophotometrically and analyzed by means of suitable computer programs.

By a sophisticated serial optical intensification process to reduce random "noise" in electron micrographs Labaw succeeded in making direct photographs showing the individual shape and lattice arrangement of human

gamma globulin molecules in the crystalline state. The shape is unusual -- Y- or T-shaped -- as indicated also by x-ray diffraction.

Murayama investigated the effects of the halides of five monovalent metallic cations on aggregation of sickle cell hemoglobin. He found only NaI to possess a delaying effect in which also it appears to act synergistically with urea. Murayama has also found a diffusible, heat-stable co-factor, possibly Mg ion activated, which is necessary for aggregation of purified sickle cell hemoglobin.

F. Hagins has found squid rhodopsin to have a m.w. of 60,000, to have a single n-terminal glycine and to be quite different in amino acid composition from vertebrate rhodopsin.

Biophysical Coupling Processes

Striated muscle shows in the electron microscope an interdigitating array of longitudinal thick (myosin) and thin (actin) microfilaments. When a muscle "contracts" these filaments slide past each other, increasing their mutual overlap rather than shortening. According to the Huxley theory of the contraction process, the motive force for the shortening of the muscle fiber is the attachment and shortening of numerous minute "cross bridges" between thick and thin filaments. The quantitative aspects of contraction are thought to depend on three variables, the rate of making cross bridges, the rate of breaking cross bridges and the force exerted by a single cross bridge under strain. Pitts has measured the force and velocity transients when single tetanized muscle fibers under tension are released in one or in two steps. Nolan and Podolsky have matched the empirical data with computer-modeled equations, using new values for the Huxley variables. From these studies it has been concluded that the "reach" of a cross-bridge is at least 100 Å, that the force exerted by a cross bridge is unequal on the two sides of its nul position and that the number of cross bridges formed increases when the velocity of shortening increases.

W. Hagins and collaborators have continued their study of the electrophysiology of the visual process. Studying current flow in the distal segment of the retinal rod during illumination, Hagins and Rüppel found that the short-lived "fast photovoltage", arises from a photochemically induced charge displacement in the rod membrane, rather than in the central, rhodopsin-bearing portion. By computerized cable theory analysis of rod membrane capacitance and conductance changes during the photovoltage it was found that most of the infolded membranous discs of the rod outer segments are electrically isolated from the plasma membrane. Yoshikami, Hagins and Rüppel found that illumination, application of 10 mM Ca^{++}, or removal of external NA^{+} all increase rod-membrane resistance. These effects are interpreted as suggesting that Ca^{++} may control retinal excitation by controlling Na-permeable channels in the rod membrane.

Studying the effects of visual input on the central nervous pacemaker for flashing in the firefly, Buck and collaborators have found that the brain adds a fixed amount of central delay to the effector-triggering delay whenever the eye is properly illuminated. The effect is interpreted as a re-

setting of a timing circuit which behaves like a relaxation oscillator.

Physical Chemistry of Membranes and Polymers

Sollner and Shean have measured the concentration potentials arising across strong base liquid membranes, consisting of various dielectric solvents and several different ion exchanger compounds at various concentrations in cells with solutions of various electrolytes in a wide concentration range. Within a wide set of conditions, the potentials obtained virtually reach the theoretically calculated maxima, deviation being well understood and qualitatively predictable. Bi-ionic potentials arising across such membranes were unexpectedly high, up to 400 mvolt with common inorganic anions. Studying porous membranes they found that the anomalies in the relationship between the electrical resistances and rates of self-exchange of ions across quaternary ammonium polyelectrolyte activated collodion matrix membranes are eliminated by treatment with aqueous alcohol solutions and return if the membranes are redried.

Lecithin, a component of some biological membranes, swells in water to form a lamellar structure in which water layers alternate with bimolecular layers of the phospholipid. In collaboration with Dr. Eanes of NIDR Gottlieb has found by low angle x-ray diffraction measurements that the swelling of the lecithin-water complex is promoted by electrolytes in the order KCl > NaCl > HCl > Na_2SO_4 > no electrolyte LiCl > $CaCl_2$, and the thickness of the bimolecular layers in the order HCl > $NaCl_2$ = no electrolyte > KCl = NaCl = LiCl = Na_2SO_4. These results are thought due to weakening of inner lecithin-lecithin salt bonds and specific ionic interactions.

Intermolecular energies of condensed monomolecular lipid films spread on water have been studied by Gershfeld and Pagano. They find from internal energy measurements that the lipid region behaves like bulk hydrocarbon liquid and can be treated as in liquid-expanded or liquid-condensed monolayer states. Polar groups were found to contribute to the internal energy of the films and to the ordering of the water structure.

Growth and Metabolism

Kempner succeeded in constructing a completely defined minimal growth medium for the protozoan Euglena. Free mannitol was found in the Euglena cell and the very basic dipeptide arginyl-glutamine has been identified in collaboration with Levenbook. This and other small peptides are metabolically active and derived from glutamic acid, the sole carbon source.

Levenbook and Bauer have completed their study of aldolase as a model for protein synthesis during insect development and metamorphosis. The most refined techniques (computerized statistics of acrylamide gel electrophoresis results, isoelectric focusing) failed to establish any except quantitative difference between any of the 5 isozymes of muscle aldolase in larval and adult blowflies. Fat body aldolase is different from muscle aldolase, more difficult to purify, not resolvable into subspecies, and possibly not identical in larva and adult.

Park, continuing her study of budding in Hydra, found that the fact that the bud of H. pseudoligactis takes 2-3 times as long to develop as in H. littoralis is due to delay in detachment, not to differing rates of tissue movement or differentiation.

Altland studied the effects of cigarette smoke on metabolism and survival of rats in various concentrations of O_2. All rats survived for at least 4 hours in a 11.8% O_2 atmosphere containing tobacco or corn silk smoke and a carbon monoxide concentration of .045% (COHb 27%). At lower O_2 concentrations fatalities occurred, with the tobacco smoke contributing substantially to the mortality since rats given .045% CO in 8.3% O_2 in absence of smoke had a lower death rate than the rats in tobacco smoke. It was found that anaerobic metabolism was accelerated (excess lactate) and that severe hypothermia was produced by the tobacco smoke.

Altland and Dieter found that prolonged physical exertion in a cold environment was readily tolerated by animals previously acclimated to cold, as evidenced by their ability to increase muscle metabolism and other homeostatic adjustments, whereas many of the unacclimated animals were unable to elevate intermediary metabolism in the muscles, exhibited a marked loss of enzymes to the serum and succumbed to the dual stress of prolonged exercise in the cold.

Dieter studied a thymic humoral factor in guinea pigs, a non-steroidal hormone which stimulates the growth and cell-mediated immune responses of peripheral lymphoid organs. Production depended on the concentration and oxidation state of vitamin C in the thymus. Lympholytic steroid hormone treatment of rats or cockerels decreased the concentration of vitamin C in their thymuses, and altered the oxidation state of the vitamin so that the remaining portion was largely dehydroascorbic acid.

Serial No. NIAMD-LPB-1
1. Physical Biology
2. Cellular Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Mechanism of Muscular Contraction

Previous Serial Number: Same

Principal Investigator: R. J. Podolsky

Other Investigators: M. Schoenberg, A. C. Nolan, W. R. Pitts, Jr.

Man Years

Total:	5
Professional:	4
Other:	1

Project Description:

Objectives:

1. To work out the molecular mechanism of muscular contraction.
2. To understand the control processes for contractility.

Methods Employed:

1. Analysis of the motion of both intact muscle fibers and "skinned" fiber segments under chemically controlled conditions.
2. Study of the electrical and chemical parameters involved in the activation of various types of muscle fibers.
3. Measurement of ionic fluxes across the internal membranes of skinned muscle fibers under various conditions.

Major Findings:

1. The response of muscle fibers to both single and double force steps directly reflects the kinetic parameters associated with the making and breaking of cross-bridges between the two types of myofilaments.
2. The average cross-bridge stroke during muscle contraction is at least 100 Å.
3. The force function of the cross-bridges is asymmetric.
4. The number of cross-bridges present at any moment increases when shortening velocity increases.

Significance to NIAMD Research:

The elucidation of the molecular mechanism of muscular contraction, together with the chemistry of the activation process, can be useful in the rational handling of neuromuscular and cardiovascular disease.

Proposed Course of Project:

1. The influence of various agents (pCa, pH, ionic strength, metal ions, ATP) on the ability of muscle fibers to develop force and to shorten will be examined directly in skinned fiber segments.
2. The influence of these same agents on myofilament spacing and possible cross-bridge configuration will be examined in skinned fibers by X-ray diffraction.
3. The mechanical response of muscle fibers to controlled changes in load will be compared with the motion predicted from a cross link contraction model with previously characterized properties.
4. Movement of calcium between the myofilament space and the sarcoplasmic reticulum will be studied by tracer methods.

Publications:

Podolsky, R. J., and Teichholz, L. E.: The relation between calcium and contraction kinetics in skinned muscle fibres. J. Physiol. 211:19-35 (1970).

Podolsky, R. J., and Nolan, A. C.: Cross-bridge properties derived from physiological studies of muscle fibers. In Podolsky, R. J. (Ed.): Contractility of Muscle Cells and Related Processes. Englewood Cliffs, N. J., Prentice-Hall, in press.

Serial No. NIAMD-LPB-2
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Triggering of Bioluminescence. Sense of Rhythm.

Previous Serial Number: NIAMD-LPB-3

Principal Investigator: John B. Buck

Other Investigators: Dr. Thomas A. Hopkins; Mr. Charles H. Hanna

Cooperating Units: Mrs. Elisabeth M. Buck, Guest Worker; Dr. Frank A. Hanson, University of Texas, Houston, Texas; Dr. James F. Case, Univ. of Calif., Santa Barbara, Calif.; Mr. Roger Halvorson, Univ. of Calif., Santa Barbara, Calif.; Mr. Darwin Tiemann, China Lake, California.

Man Years:

Total:	5.0
Professional:	3.0
Other:	2.0

Project Description:

Objectives:

To understand how action potentials in nerves are coupled to chemical light production in luminous organs. To understand how individual central nervous oscillators can become mutually entrained.

Methods Employed:

Standard biochemical and spectroscopic; pharmacological; psychophysical; electron microscopy.

Major Findings:

1. Single male New Guinea Pteroptyx fireflies can duplicate both faster and slower rhythms of light. Since the interval between the pacer flash and the firefly flash of the next cycle equals the firefly's normal free-run period it is suggested that the pacer signal resets the flash-timing oscillator in the brain, thus providing a mechanism for synchronization. (with E. Buck, Hanson, Case)

2. The luminescence of the Brazilian "railroad-worm" Phrixothrix was tested by perfusions with noradrenaline, amphetamine, acetyl choline and eserine and judged to be under adrenergic control. (with Halvorson, Case)

3. Photocyte granules in photophores from midwater myctophid fish and in fireflies were found to have considerable ultrastructural similarity to the peroxisomes of green plant cells, a similarity of considerable theoretical interest in view of the postulated involvement of hydroperoxides in firefly bioluminescence. (Hanna)

4. Luminescence in Phengodidae, Zarhippus, and Phrixothrix (non-Lampyrid Coleoptera) results from the same product excited state as in fireflies both in vivo and in vitro, as judged by biochemical and spectroscopic methods. A unique emitter, not seen in fireflies, is responsible for the red luminescence of the "railroad-worm" head light organ.

Significance to NIAMD Research:

Information about excitation-luminescence coupling should contribute to the understanding of energy transduction in general. It is hoped that the behavioral work will be directly applicable to pacemaking and rhythmic entrainment in man, since human beings and certain fireflies are alike (and different from practically all other animals) in being able to synchronize.

Proposed Course of Project:

No major changes anticipated.

Honors:

Executive Committee, Marine Biological Laboratory, Woods Hole, Massachusetts.

Publications:

McLean, Miriam, Buck, John, and Hanson, Frank E.: Culture and larval behavior of photurid fireflies. Amer. Midland Naturalist. In press.

REPORTED IN PRESS - 1970

Peterson, Margaret K.: The fine structure of the larval firefly light organ. J. Morphol. 131: 103-115, 1970.

Serial No. NIAMD-LPB-3
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Aldolase in the Blowfly Phormia regina.

Previous Serial Number: NIAMD-LPB-4

Principal Investigator: L. Levenbook

Other Investigators: A. Bauer

Cooperating Units: R.F.N. Hutchins (formally of U. S. Dept. of Agriculture); Ellis Kempner (NIAMD-LPB-22); W. A. Hagins (NIAMD-LPB-11)

Man Years

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

1. To characterize and compare purified muscle and fat body aldolases from larval and adult blowflies.

Methods Employed:

Routine protein purification procedures and a variety of chemical physical techniques. Special emphasis on gel-electrophoresis and iso-electric focusing.

Major Findings:

1. Muscle. A sophisticated statistical analysis employing electrophoresis on acrylamide gels of known porosity and a computer program has revealed that with respect to molecular weight, molecular radius, free mobility and valence, larval and adult blowfly muscle aldolases are indistinguishable. Iso-electric focusing has shown further that muscle enzyme from larvae and adults is composed of five active species with the same iso-electric points at about pH 7.0, 7.2, 7.4, 7.7 and 7.9. (The values presented for last year's report were in error). Although differences in the amounts of corresponding species were evident, the identity of these two homologous proteins from the two developmental stages appears to be proven.

2. Fat body. (Identity of homologous proteins from larval and adult fat body is a more stringent test of the uniqueness of the fly genome than are muscle proteins; it is for this reason that efforts have continued with this recalcitrant tissue). Purification of mg. amounts of fat body aldolase has still defied all our efforts. Enzyme from these tissues could not be resolved into sub-species (although these presumably must be present), nor could larval and adult enzymes be satisfactorily distinguished by either iso-electric focusing or by gel electrophoresis employing a wide range of buffer species. There are some indications that larval and adult fat body enzymes are not identical, but corroborative evidence is still required.

3. In collaboration with Dr. E. Kempner, LPB, a number of peptides has been recognized in Euglena, and one of them has been isolated and identified as arginyl-glutamine.

4. In collaboration with Drs. W. and F. Hagins, squid rhodopsin has been purified, and its N-terminal residue, minimum molecular, weight and amino acid composition determined.

5. Three manuscripts and a book review have been written.

Significance to NIAMD Research:

The present work is envisaged as shedding light on two fundamental questions: (1) to what extent intact proteins in a developing organism can be re-utilized as such (i.e. without prior breakdown to free amino acids or small peptides). (2) Is an organism that undergoes drastic metamorphosis equipped with one, or two or more sets of genetic information such that it can make only one type of a particular protein, or a different type of homologous protein depending upon developmental stage.

Proposed Course of Project:

Definite termination of this program by the summer. A new phase of research will be initiated concerning the structure and biological role of calliphorin, the major soluble larval protein of most higher Diptera. Continuation of collaboration with Drs. Hagins and possibly Dr. Kempner.

Publications:

Levenbook, L. and Krishna, I.: Effect of ageing on amino acid turnover and rate of protein-synethesis in the blowfly Phormia regina. J. Insect Physiol. 17: 9-12, 1971.

Levenbook, L.: Polysaccharide-protein complexes in invertebrates. (Book review) Quart. Rev. Biol. 45: 388, 1970.

Levenbook, L., Hutchins, R.F.N. and Bauer, A.C.: Uric acid and basic amino acids during metamorphosis of the tobacco hornworm, Menduca sexta, with special reference to the meconium. J. Insect Physiol. In press.

Serial No. NIAMD-LPB-4
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Sickle Cell Disease: Molecular Aspects of Human Red Cell Sickling and Unsickling

Previous Serial Number: NIAMD-LPB-5

Principal Investigator: Makio Murayama

Other Investigators: Dr. Shiro Ohnoki (from 23 February 1971)

Cooperating Units: None

Man Years

Total:	1 1/3
Professional:	1 1/3
Other:	0

Project Description:

Objectives:

1. To find a molecular mechanism of unsickling of sickled erythrocytes from sickle cell anemia patients.

2. Ultrastructure of the sickled human erythrocyte.

3. To find out the nature, origin, and metabolism of dialyzable sickle cell hemoglobin aggregation factor, the Hb S co-factor.

Methods Employed:

Aggregation-deaggregation of sickle cell hemoglobin by pressure, temperature and alkali halides. Freeze etching, ion etching and scanning electron microscopy.

Major Findings:

It has been known for some time that sodium iodide solubilizes so-called urea-soluble fibrin clots. It occurred to us to find out if sodium iodide (and other alkali metal halides) might unsickle and deaggregate Hb S polymer. We investigated the influence of lithium, sodium, potassium, rubidium and cesium halides on the rate of aggregation of purified sickle

cell hemoglobin. We found that sodium iodide alone slows the rate of aggregation of Hb S, and the mode of action is probably due to a non-specific ion effect. Sodium iodide appears to act synergistically with urea, i.e., the unsickling by an equimolar mixture of urea and sodium iodide appears to be nearly twice as great as by an equimolar solution of either urea or sodium iodide alone.

In scanning electron micrographs of sickled erythrocytes' inner structure by removing the outer structure by controlled ion-etching (argon beam, accelerated at 4 KEV for 4 minutes at 7×10^{-4} torr.). It can be seen that the tip of each rod has been partly etched away but a diameter measured at the base is about 900 A, suggesting that there are bundles of microtubules or filaments forming large fibers.

Highly purified sickle cell hemoglobin does not aggregate at all. However, when a small amount of the residue from the diffusate is added back, the aggregation takes place dramatically; this suggests that there is an aggregation co-factor for sickle cell hemoglobin. This substance appears to be heat stable. However, its activity vanishes after (nitric acid) ashing, suggesting that it may not be a metallo ion. However, the activator for the Hb S co-factor appears to be magnesium ion.

Significance to NIAMD Research:

As stated previously, sickle cell anemia is a hereditary hemolytic anemia; it is one of the best studied and best understood of all human anemias; it is a metabolic disease as well as a molecular disease. In March 1971 Linus Pauling said, "Sickle-cell disease is the first disease to have become thoroughly understood, the first disease for which a clear and complete explanation of the manifestation could be formulated. This understanding of the nature of the disease has led, through the efforts especially of Dr. Makio Murayama and Dr. Robert M. Nalbandian, to the development of a method of controlling the crises of the disease. Sickle cell anemia thus has become the first disease for which there is a known molecular basis for treatment."

Proposed Course of Project:

The molecular mechanism of unsickling of sickled erythrocytes will be investigated further from the standpoint of thermodynamics.

Isolation, identification, and characterization of the dialyzable aggregation co-factor for sickle cell hemoglobin will be extended.

Methods for preservation of human blood platelets will be explored.

Honors and Awards:

Foreign Travel Award (to the International Congress of Hematology) from Foundation for Research and Education in Sickle Cell Disease.

Publications:

Murayama, M.: Molecular mechanism of human red cell sickling. In Nalbandian, Robert M. (Ed.): Molecular Aspect of Sickle Cell Hemoglobin: Clinical Application. Springfield, Ill., Charles C. Thomas, 1971, pp. 3-19.

Murayama, M.: The chemical and the three-dimensional structure of human hemoglobin. Annals of Clinical Sciences. In press.

Murayama, M.: Peptide patterns ("Fingerprinting") of hemoglobin. Annals of Clinical Sciences. In press.

Altland, P. D., Brubach, H. F., Parker, M. G., Dieter, M. P., and Murayama, M.: Effects of smoke on tolerance of rats to hypoxia. J. Appl. Physiol. In press.

Serial No. NIAMD-LPB-5
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Hydroid Physiology

Previous Serial Number: NIAMD-LPB-6

Principal Investigator: Helen D. Park

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To study basic mechanisms (cell movement and cell differentiation) in morphogenesis during budding and regeneration in Hydra.

Methods Employed:

Those previously reported and in addition the use of time lapse photomicrography to follow at 50 times magnification (1) the development of a bud and (2) the reorganization and reconstitution of a short segment of body tissue into a whole hydra.

Major Findings:

1. I have continued the comparative study of budding and regeneration in H. littoralis and H. pseudoligactis. Many of the experiments done were replicates of those reported last year. There are now enough data so that they can be analyzed statistically. These additional data do not alter the conclusions reported last year.

   In addition, the following can be reported. It has been known for some time that the development of a bud (from its initiation to its detachment) takes 2-3 times longer in H. pseudoligactis than in H. littoralis. Observations of timed photographs taken of the

two species simultaneously show that both develop (elongate and differentiate hypostome, tentacles, peduncle and pedal disk) at the same rate. The difference lies in the longer time taken for detachment of the fully developed bud from the parent in H. pseudoligactis. It would have been more exciting to find that the difference between the two species is in their rate of tissue movement or differentiation.

2. Goodwin and Cohen (1970) described a phase-shift model for the spatial and temporal organization of developing systems. They reported that some preliminary experimental results on Hydra littoralis tend to confirm the model. The experiment was as follows. Periodic electrical stimulation was delivered locally by means of a thin platinum wire passing through the tissue of a regenerating animal.

   I now quote "The stimulation results in the induction of a secondary foot toward the distal end of an isolated mid-gastric region of hydra." In view of the fact that no controls were mentioned, my skepticism of the authors interpretation of the result was boundless. One obvious control is to set up the experiment as Goodwin and Cohen did, but not deliver the periodic electrical pulse through the platinum wire. This I did, with the result that a secondary foot developed toward the distal end of the segment. Therefore, periodic electrical stimulation is not necessary for the induction of a secondary foot. The results of the Goodwin and Cohen study and mine, give few leads for further study of pattern regulation in Hydra. However, two possibilities come to mind: (a) A peduncle and foot developed in abnormal position as a result of the wound held open for 24 hrs at the site of penetration of the wire through the tissue. (b) Anoxic condition of a localized group of cells caused by the weight of the wire holding the cells closely apposed to the bottom of the dish.

   An experimental approach to (a) was not attempted. The results of experiments with regard to (b) were "negative", perhaps because of technical difficulties.

3. Considerable work has been done by others in trying to reverse polarity in Hydra, i.e., in trying to induce a head at the basal end or a foot at the apical end. In our attempt to reverse polarity by grafting a peduncle and foot to the apical end of a mid-body segment we did not succeed in polarity reversal. However, we did obtain an apparent induction of a secondary peduncle and foot near the apical end of the mid body segment

Significance to NIAMD Research:

This work contributes to the basic biology of the mechanisms of growth and cell differentiation.

Proposed Course of Project:

The project will be discontinued after June 1, 1971, because of my retirement May 28, 1971.

Honors and Awards:

I have given two seminars for students at Montgomery College.

Publications:

Park, H. D., Ortmeyer, A. B., and Blankenbaker, D. P.: Cell division during regeneration in Hydra. Nature 227: 617-619, 1970.

Serial No. NIAMD-LPB-6
1. Physical Biology
2. Electrochemistry and Colloid Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Physical Chemistry of Lipids

Previous Serial Number: NIAMD-LPB-7

Principal Investigator: M. H. Gottlieb

Other Investigators: None

Cooperating Units: Dr. E. D. Eanes, NIDR, and Dr. M. Lewis, NEI

Man Years:

Total:	2/3
Professional:	2/3
Other:	0

Project Description

Objectives:

To understand the interactions between lipids, water, and electrolytes at the lipid-water interface.

Methods Employed:

X-ray diffraction, ultracentrifugation

Major Findings:

Lecithin swells in water to form a lamellar structure in which water layers alternate with bimolecular layers of the phospholipid. Low angle x-ray diffraction measurements show that the maximal swelling is a function of electrolyte in the order KCl > NaCl > HCl > Na_2SO_4 > no electrolyte > LiCl > $CaCl_2$; the electrolyte order in effect on thickness of the bimolecular layers is HCl > $CaCl_2$ = no electrolyte > KCl = NaCl = LiCl = Na_2SO_4. This can be interpreted in terms of a weakening of inner salt bonds between adjacent lecithin molecules by all electrolytes, and specific interactions between ions of the electrolyte and the ionic groups of the lecithin. This work was done in collaboration with Dr. E. D. Eanes (NIDR).

Sedimentation equilibrium studies on solutions of lysolecithin indicated no effects of NaCl, Na_2SO_4 or $CaCl_2$ on the size of the micelles formed by this phospholipid. This work was done in collaboration with Dr. M. Lewis (NEI).

Proposed Course of the Project:

The study of the swelling of lecithin will be extended to include the role of electrolyte concentration, and determination of the distribution of electrolyte between the lecithin gel and the solution phase in equilibrium with it. Similar studies are planned for the non-ionic lipids, glycerol monoesters. The effects of electrolytes on the critical micelle concentration of lysolecithin will be determined.

Significance to NIAMD:

An understanding of the lipid-electrolyte solution interface contributes to the understanding of ionic processes occurring at cell membrane surfaces and also of the lipid-protein interactions in lipoproteins.

Honors and Awards: None

Publications:

Gottlieb, M. H.: On the Rates of Exchange Between Free and Bound Counter-Ions in Polyelectrolyte Solutions I, II, and III, J. Phys. Chem., in press.

Serial No. NIAMD-LPB-7
1. Physical Biology
2. Electrochemistry and Colloid Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The physical chemistry of membranes and complex membrane systems of biological interest

Previous Serial Number: NIAMD-LPB-8

Principal Investigator: Karl Sollner

Other Investigators: Gerald M. Shean, Melvin Gottlieb, Vedoster Ingram

Cooperating Units: None

Man Years:

Total	4-1/3
Professional:	3-1/3
Other:	1

Project Description:

Objectives:

The physico-chemical study of liquid ion exchange membranes, permselective ion-exchange membranes, other membrane systems and models with the ultimate aim of providing a rational basis for various complex membrane phenomena in living systems.

Methods Employed:

Conventional chemical-analytical and electrochemical methods, especially potentiometric measurements as reported previously.pectra in areas

Major Findings:

Liquid Membranes

On the basis of the improved understanding of the basic physical chemistry of liquid ion exchanger membranes reported last year, systematic quantitative studies of the electromotive behavior of anion exchange membranes of this type were carried out under more carefully controlled experimental conditions than usual in this field. Concentration cells with membranes based on seven solvents of different dielectric constants between 2.6 to 27.4 and eight different strong base ion exchanger compounds were studied.

The loading of the membranes with ion exchanger compounds was varied over a range from 2×10^{-4} N to 2×10^{-1} N. Cells with seven different electromotively active anions in solution (SCN^-, I^-, ClO_4^-, Br^-, NO_3^-, Cl^-, IO_3^-) were studied, in some instances over a 1000 fold concentration range, 0.001/0.002 N to 1.0/2.0 N. The concentration potentials arising in these cells closely approach the theoretical maximum values over a wide range of experimental conditions. Significant deviations arise only under predictable, well understood conditions.

Bi-ionic potentials, BIP, that is the potentials arising across membranes of extreme ionic selectivity in cells like the anionic system $A^+L^-c_1 \mid \underset{\text{Membrane}}{\text{Exchanger}} \mid A^+M^-c_1$ were surveyed. These potentials are constant (± 2%) over a 1000 fold range of loading of the membranes with ion exchanger compound and practically independent of the solution concentrations. They are much higher than those observed with porous membranes. As with the porous membranes they are additive.

Porous Membranes

The investigation of the large deviations from the theoretical relationship between the electrical resistances and the rates of self-exchange of ions across collodion matrix membranes activated by adsorption of quaternary ammonium polyelectrolytes was continued. The deviations decrease, and in some instances disappear, on treatment of the membranes with aqueous ethanol solutions; the anomalous behavior returns if the membranes are redried.

Proposed Course of the Project

Liquid Membranes

Additional systematic studies of bi-ionic potentials in cells with anion exchanger membranes and a systematic study of poly-ionic potentials, potentials in cells with mixed electrolyte solutions with such membranes, will be made.

Porous Membranes

To continue the essentially empirical approach to the cause of the large deviations from the theoretical relationship between electrical resistances and rates of self-exchange of ions with the quaternary ammonium polyelectrolyte membranes.

Significance to NIAMD Research:

Movement of solvent and solutes is an important aspect of all life processes. This project tries to provide solidly founded, nonspeculative physico-chemical background information on the functional behavior of membranes in general. At the same time, these membrane studies have furnished a new, now widely used tool for the electrometric determination of ion activities - the use of permselective membranes as membrane electrodes. It is perhaps also of interest that Sollner's "permselective" membranes are the prototypes of the ion exchange membranes which are used now in the electrodialytic desalination of sea and brackish water.

Publications:

Sollner, K.: The basic electrochemistry of liquid membranes, Proceedings of the Thomas Graham Centennial Symposium of Diffusion Processes, September 22-24, 1969, University of Strathclyde, Glasgow, Scotland. London, Gordon and Breach, 1970, pp 655-730. (Reported last year as "In Press")

Serial No. NIAMD-LPB-8
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:

1. Determination of the physical and chemical characteristics of complexes between retinal and phosphatidyl ethanolamines.

2. Construction of a model system to approximate the complex described and to determine a possible relationship to rhodopsin.

Previous Serial Number: NIAMD-LPB-9

Principal Investigator: Ralph G. Adams

Other Investigators: W. H. Jennings
N. E. Sharpless

Cooperating Units: None

Man Years:

Total:	1-2/12
Professional:	1-2/12
Others:	None

Project Description:

Objectives:

1. Complexes between phosphatidyl Ethanolamine (P.E. and retinaldehyde form spontaneously in vitro but only in anhydrous organic solvents. If these complexes are, as we believe, the chromophoric portion of the visual pigment rhodopsin, then they must exist in an aqueous surrounding. A thorough understanding of the structure and kinetics of the formation of the complexes is prerequisite to understanding how they exist in an aqueous environment. It is likely a hydrophobic layer surrounds the complex and its removal produces destruction of the latter causing "bleaching."

2. An obvious endpoint to seek is the construction of synthetic rhodopsin or functional analog. To accomplish that purpose physical, quantum mechanical and chemical models have been constructed and tested.

Major Findings:

1. After firm establishment of the structure of the phospholipid-retinal complex by virtue of model complexes reported previously, we attempted to define the kinetic course for the formation of complexes I and II. The difficulty lay in the overlapping spectra with maxima at 390 nm for retinal, 455 for complex I, and 500 for complex II, all broad and further complicated by a large peak at 330 nm which we were unable to explain. By a series of manipulations of spectra, recorded on line by the 516 Honeywell computer, we have been able to separate peaks and determine molar extinction coefficients. Recently we have explained the peak absorption at 330 nm as the unprotonated Schiff's base precursor of complex I. It has a low extinction and is favored by addition of H^+.

We have submitted for publication results we feel justify considering Complex II as the chromophore of rhodopsin.

2. A successful duplication of rhodopsin action in vitro still has not been achieved but in view of recent findings about the formation of Complex II (above) and use of the computer to eliminate false avenues, we are hoping for success.

Significance to NIAMD Research:

To elucidate the structure of rhodopsin, the visual pigment is an obviously important step in understanding vision.

Recent publications by others have been critical of the concept of phospholipid-retinal complexes as part of the rhodopsin molecule. We believe the interpretation of these is in error. Our study of the spectral characteristics of rhodopsin will, hopefully, lead to a reinterpretation.

Proposed course:

1. Application of results of kinetic studies to formation of model compounds approximating rhodopsin.

2. Flash photolysis of model compounds of retinal and phospholipid

3. Further experimentation with synthetic rhodopsin solutions.

4. Analysis of native rhodopsin in light of data now available concerning complexes.

Honors and Awards: None

Publications:

Sharpless, N.E., Adams, R. G., and Jennings, W. H. A molecular model for a phospholipid-retinaldehyde complex absorbing at 500 nm. Photochem. Photobiol. 13, 21 (1971).

Serial No. NIAMD-LPB-9
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Molecular structure determined by spectroscopic methods

Previous Serial Number: NIAMD-LPB-10

Principal Investigator: Edwin D. Becker

Other Invesitgators: Robert B. Bradley
Regitze R. Shoup
Herman J. C. Yeh

Cooperating Units: Marjorie S. Malmberg (NIH Fellow)
Edward Tucker (Air Force Fellow)
H. T. Miles, T. Lewis (NIAMD-LMB)
T. C. Farrar, D. L. VanderHart (National Bureau of Standards)
S. Druck (Catholic University)
T. Axenrod (City University of New York)
M. Schwartz (Agriculture Research Center)

Man Years

Total:	4
Professional:	3
Other:	1

Project Description:

Objectives:

1. Development of an understanding of the forces within and between molecules, especially those of potential biological importance.
2. Development of spectroscopic methods for studying molecular structure and analyzing materials of chemical and biological interest.

Methods Employed:

The primary techniques used in this work are infrared spectroscopy (IR), Raman spectroscopy, and nuclear magnetic resonance spectroscopy (NMR). The three methods supplement each other in providing detailed information about the structure of molecules. In addition, the spectra are highly sensitive to the effects of molecular interactions. For studies of hydrogen bonding high precision vapor pressure measures provide information complementary to that obtained by the spectroscopic techniques.

Major Findings:

Our major effort during the past year has been directed toward the elucidation of relaxation mechanisms for ^{13}C nuclei and the further development of Fourier transform methods for studying ^{13}C and other nuclei of low sensitivity. We have submitted for publication a preliminary communication describing the ^{13}C relaxation behavior in seven molecules as functions of temperature and in some cases of frequency. From these data we have inferred the contributions of four different mechanisms to the relaxation process. (Shoup, Becker, Druck, Farrar)

Further studies of the utility and limitation of the driven equilibrium Fourier transform (DEFT) method described last year have been carried out with several ^{13}C-enriched compounds. A very important and completely unexpected finding was that for ^{13}C spin-coupled to ^{1}H, the spin-spin relaxation time, T_2 (^{13}C), is not equal to T_1 (^{13}C), the spin-lattice relaxation time, but is governed primarily by T_1 (^{1}H), the spin-lattice relaxation time of the proton. This result has ramifications for line widths of ^{13}C resonance and imposes limitations on the sensitivity enhancement attainable with DEFT. (Shoup, VanderHart)

An NMR study of ^{15}N-enriched substituted anilines has been completed and submitted for publication, as have papers on $^{14}N/^{15}N$ isotope effects on nitrogen chemical shifts and a proposed scale for reporting such chemical shifts. (Becker, Bradley, Axenrod) An NMR study of ^{23}Na in muscle tissue has provided evidence for substantial differences in line width between sodium within the muscle cells and that held interstitially. (Yeh, Becker) Also completed during the year and being prepared for publication are proton NMR studies of a series of asymmetric cyanoacetates (Becker, Bradley, Schwartz); NMR studies of internal rotation in substituted cytosines (Shoup, Miles, Becker); and IR and Raman studies of 1-methyluracil (Lewis, Miles, Becker). Studies of hydrogen bonding of t-butanol in a solvent of n-hexadecane have recently been initiated. (Tucker)

Significance to NIAMD Research:

In this project we are attempting to obtain basic information on the structure and molecular interactions of nucleosides and their derivatives and to learn more about the nature of hydrogen bonding in these and other molecules. Such information may aid in an understanding of the relation between structure and function of nucleic acids and enzymes. In addition, the development of spectroscopic techniques applicable to molecules of biological activity and interest, such as steroids, alkaloids, pigments, etc., aids in the structure elucidation of many natural and synthetic substances studied throughout NIH.

Proposed Course of Project:

1. We expect to complete the construction and testing of a 55 MHz pulsed NMR system with which we can study ^{13}C at 52 kgauss (superconducting solenoid), as well as ^{1}H at 13 kgauss fields.

2. We shall complete our tests of the utility of DEFT and related methods.

3. We have begun a study of spin-lattice relaxation in a series of hydrocarbons, which will lead to similar investigations in appropriate lipid systems and polypeptides.

4. We expect to complete ^{15}N double resonance studies of ^{15}N-enriched nitroso compounds and to investigate spin-lattice relaxation in other ^{15}N-enriched compounds.

5. We plan to study hydrogen bonding in alcohols by the complementary methods of vapor pressure determination, NMR and IR, and to use the first two techniques to investigate hydrogen bonding and "stacking" interactions in nucleosides.

Honors and Awards:

Washington Academy of Sciences Award in the Physical Sciences, 1971.

Publications:

Paolillo, L. and Becker, E. D.: The relative signs of the spin-spin coupling constants in $CH_3{}^{15}NH_2$. J. Mag. Resonance 3: 200-203, 1970.

Shoup, R. R. and VanderHart, D. L.: Effect of CH scaler coupling on ^{13}C transverse relaxation times. J. Amer. Chem. Soc. In press.

Becker, E. D., Bradley, R. B. and Axenrod, T.: Double resonance studies of isotope effects on nitrogen chemical shifts. J. Mag. Resonance. In press.

Becker, E. D.: A proposed scale for nitrogen chemical shifts. J. Mag. Resonance. In press.

REPORTED IN PRESS - 1970

Cramstad, T. and Becker, E. D.: NMR studies of hydrogen bonding in phenol with several proton acceptors. J. Mol. Structure 5: 253-261, 1970.

Paolillo, L. and Becker, E. D.: The effect of solvent interactions and hydrogen bonding ^{15}N chemical shifts and ^{15}N-H coupling constants. J. Mag. Resonance. 2: 168-173, 1970.

Jerina, D. M., Boyd, D. R., Paolillo, L., and Becker, E. D.: Stereospecific chemical shifts and coupling constants in ^{15}N-oxaziridines. Tetrahedron Letters 18: 1483-1484, 1970.

Serial No. NIAMD-LPB-10
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Molecular structure, organization and intermolecular forces

Previous Serial Number: NIAMD-LPB-11

Principal Investigator: Elliot Charney

Other Investigators: William A. Eaton
Julie B. Milstien

Cooperating Units: Dr. Marvin Makinen (NIDR-LB)
Dr. Thomas Lewis, City University of New York
Dr. Walter Lovenberg (NHLI-ETB)
Dr. Graham Palmer, University of Michigan
Dr. E. D. Rossomando, Brandeis University
Dr. Lin Tsai (NHLI-LB)
Dr. Kiwamu Yamaoka, Hiroshima University, Japan

Man Years:

Total:	4
Professional:	3
Other:	1

Project Description:

The general objectives of this research are to determine the role of molecular structure and of intermolecular forces in chemical, physical and biological phenomena by exploring the electrical (dipole moment and electric dichroism) and optical (spectroscopic absorption, optical rotatory dispersion, dichroism) properties of molecules.

Methods Employed:

The methods have all been described in previous reports including the methods which are unique or almost unique to this laboratory.

Major Findings:

1. Electric field induced dichroism of large molecules

   a. Theory (with Dr. Yamaoka): The theory of the orientation of large macromolecules in both weak and strong electric fields and the effect of this orientation on the absorption of plane polarized light propagated in the oriented system has been completed.

b. Polynucleotides (with Dr. Milstein): There is a small but significant increase in the relaxation time and in the dichroic signals of polyriboseadenylic acid as ionic strength is decreased at the same applied field strength. This can only come from increase in the axial ratio, thus an extension of the poly A rod either from a change in tertiary structure or from a stiffening or straightening of the rod. An increase caused by a change in the tertiary structure would imply a tilting of the bases and a decrease of the dichroism, while the stiffening would result in an increase of the dichroism. Preliminary results favor the latter.

c. Hemoglobin-haptoglobin and f1 phage systems. In the complex formed between human blood glycoprotein, haptoglobin, and human hemoglobin preliminary studies indicate that the heme moiety may not be held rigidly to the haptoglobin (Makinen, Milstien). In the f1 phage system, the dichroism data on specific mutants of a minor coat protein, shows that the so-called A-protein controls the arrangement of the major coat protein of the f1 phage and its mutants (Rossomando, Milstien).

2. Optical activity and electronic states of small molecules

a. α-Diketones (with Dr. Tsai): The optical activity of camphorquinone has been shown to arise from the dissymmetry of the diketone chromophore and the four lowest excited electronic states have been identified. We have confirmed experimentally the recent theoretical prediction that in these compounds the highest filled molecular orbital is better represented by the symmetric combination of the non-bonding orbitals of the oxygen than by the antisymmetric combination as had been believed since the 1940's when the first quantum-mechanical theories were applied to these systems.

b. Cytosine monohydrate. A polarized single crystal absorption study on cytosine monohydrate has been completed. With this work experimentally-determined transition moment directions are now available for derivatives of four of the five common nucleic acid bases--uracil, thymine, cytosine, adenine. For the first $\pi \rightarrow \pi^*$ transition, theoretically predicted transition moment directions agree well with experiment for uracil and thymine, but poorly for cytosine. (Eaton and Lewis)

3. Electronic states of iron containing proteins

a. Iron-sulfur proteins. A study of the near infrared circular dichroism of adrenodoxin and plant ferredoxins is being completed. These proteins play a central role in electron transfer processes. From the location of magnetic-dipole d-d transitions in the region 1.0 - 2.5 μ, it has been possible to establish that these two-iron proteins contain tetrahedrally bonded high spin iron. (Eaton, Lovenberg, Palmer)

b. Heme proteins. Polarized single crystal spectra of a variety of hemoglobin derivatives show considerable differences among the various liganded derivatives of ferrous and ferric hemoglobin, in contrast to the conclusion, from Perutz's low resolution x-ray investigation, that all liganded forms of hemoglobin have the same conformation. (Eaton and Makinen)

Significance to NIAMD Research:

Using electro-magnetic ratiation to determine some optical and electrical properties of small and large molecules helps elucidate their structures and the relationships between structure, reactivity, and the intermolecular forces.

Proposed Course of Project:

We will continue a major effort through single crystal spectroscopy and electric dichroism to discover the origin of the large angle which the electronic transition moment, supposedly in the plane of the constituent nucleic acids, appears to make with the perpendicular to the helix axes of DNA and poly A. In addition the recent acquisition of a fast signal averaging device will now permit us to examine very low molecular weight fragments of DNA and poly A, which are known hydrodynamically to be stiff rods. We also plan to use this device to investigate the structure of much smaller molecules by linear electric-dichrosim and birefringence techniques.

Publications:

Eaton, W. A. and Lovenberg, W.: Nesr-infrared circular dichroism of an iron-sulfur protein. d → d transitions in rubredoxin. J. Amer. Chem. Soc. 92: 7195-7198, 1970.

Charney, E.: Linear dichroism with special emphasis on electric field induced linear dichroism. In Cantoni, G. and Davies, D. (Eds.): Procedures in Nucleic Acid Research. New York, Harper and Row. In press.

Charney, E. and Yamaoka. K.: Electric dichroism of DNA in Solution. Proceedings of the XXIII International Congress of Pure and Applied Chemistry. In Press.

Rossomando, E. F. and Milstien, J. B.: Electro-optic evidence for the control of the structure of bacteriophage f1 by a minor coat protein. J. Mol. Biol. In Press.

Lewis, T. P. and Eaton, W. A.: Polarized single crystal absorption spectrum of cytosine monohydrate. J. Amer. Chem. Soc. In press.

REPORTED IN PRESS - 1970

Charney, E., Milstien, J. B., and Yamaoka, K.: Electric dichroism studies: poly-γ-benzylglutamate and poly-β-benzylaspartate. J. Amer. Chem. Soc. 92: 2657-2664, 1970.

Milstien, J. B. and Charney, E.: Electric dichroism studies: The interaction of trifluoroacetic acid with poly-γ-benzylglutamate. Biopolymers 9: 991-1000, 1970.

Eaton, W. A. and Lewis, T. P.: Polarized crystal absorption spectrum of 1-methyluracil. J. Chem. Phys. 53: 2164-2172, 1970.

Serial No. NIAMD-LPB-11
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Physical and Chemical Bases of Photoreception

Previous Serial Number: NIAMD-LPB-12

Principal Investigator: W. A. Hagins

Other Investigators: Shuko Yoshikami
Hartmann Rüppel
Frances M. Hagins

Cooperating Units: Kurt Heinrich, U.S. National Bureau of Standards
Gerald Wetzel, Harry Diamond Laboratory, U.S. Army Materiel Command, Washington, D. C.

Man Years

Total:	3.5
Professional:	2.5
Other:	1

Project Description:

Objectives:

To outline the successive events by which light quanta absorbed in the receptors of animals lead to the transmission of information to the brain.

Methods Employed:

1. Measurements of currents, voltages, and impedances of the retina by conventional electronic methods.
2. Measurement of photochemical changes in rhodopsin in living cells by kinetic microspectrophotometry.
3. Analysis by digital computation of electrical signals produced by photoreceptors.
4. Investigation of the chemical structure of rhodopsin by spectrophotometry and amino acid analysis.
5. Electron microscopy of the cellular structure of photoreceptors.
6. Analysis of the ionic composition of the retina by electron probe microanalysis.

Major Findings:

1. The fast photovoltage, a short, weak electrical signal produced by vertebrate retinas, has been shown to arise in the outer segments of retinal rods. It arises as a photochemically-induced charge displacement in the plasma membrane and not in the core of rhodopsin-bearing internal disks where most of the light used in seeing is absorbed. Evidently the envelope membrane also contains rhodopsin-a conclusion which harmonizes well with what is known of the way in which rod outer segments are formed. (W. Hagins & H. Rüppel)
2. Using the fast photovoltage as a method of polarizing the cell membranes of rod outer segments without need for internal electrodes, a method has been developed for estimating the membrane capacitance and conductance of rods. Fast photoelectric signals recorded from the retina are matched with theoretical curves generated by solving the differential equation for an inhomogeneous electric cable with the geometry of a retinal rod and with adjustable membrane parameters. Using this method, it has been found that the infolded membranous disks of rod outer segments are electrically isolated from the plasma membrane except for a few disks at the junction between inner and outer segments. (W. Hagins & H. Rüppel)
3. The curve-fitting procedure of 2 above has been used to measure changes in the membrane conductance of rod outer segments accompanying the physiological electrical responses of living rods. Exposure of rods to light, external application of 10 mM Ca^{++} to them or removal of all external Na^{+} ions all increase the rod membrane resistance to the same degree. The rod envelope membrane evidently contains Na-permeable channels which are normally open but are closed by an increase in the Ca^{++} activity in the rod cytoplasm. Either an experimentally increased external Ca^{++} concentration or a light-induced release of Ca^{++} from the internal disks may be able to increase the activity of cytoplasmic Ca^{++}. (S. Yoshikami & W. Hagins)
4. A structural study of squid rhodopsin has revealed that it has a molecular weight of 60,000 by gel electrophoresis, a single N-terminal glycine and an amino acid composition quite different from that of vertebrate rhodopsin. It constitutes the main membrane protein of squid rod outer segments. (F. Hagins).

Significance to NIAMD Research:

Progress in the understanding of molecular events underlying excitation of photoreceptors is obviously important in research on vision. In addition, it may provide a point of attack on the nature of permeability changes which occur in the surface membranes of electrically active cells, such as nerve and muscle, during transmission of nerve impulses and initiation of muscular contraction.

Proposed Course of Project:

Present experiments are directed towards answering the three questions:

1. What is the ionic mechanism of the currents produced by vertebrate photoreceptors?
2. What part do the kinetics of the rod responses to light play in shaping the overall function of the eye in dark and light adaptation?
3. What part do rhodopsin's photochemical reactions play in initiating the electrical responses of rods?

Publications:

Hagins, W. A., and Rüppel, H.: Fast photoelectric effects and the properties of vertebrate photoreceptors as electric cables. Fed. Proc. 30:64-68 (1971).

REPORTED IN PRESS - 1970

Hagins, W. A., Penn, R. D., and Yoshikami, S.: Dark current and photocurrent of retinal rods. Biophys. J. 10:380-412 (1970).

Serial No. NIAMD-LPB-12
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Structure of paramagnetic molecules and their interaction with the environment

Previous Serial Number: NIAMD-LPB-13

Principal Investigator: Hideo Kon

Other Investigators: Ryo Hirasawa

Cooperating Units: None

Man Years

Total:	1.7
Professional:	1.7
Other:	0

Project Description:

Objectives:

To investigate the electronic structure of transition metal ions and/or organic free radicals incorporated in some biologically interesting systems and to obtain information concerning the state of the surroundings.

Methods Employed:

Electron paramagnetic resonance (epr) was the basic experimental means and the crystal field theory the principal theoretical tool. Subsidiary methods used include the nuclear magnetic resonance (nmr), x-ray crystallography, single crystal absorption spectrometry.

Major Findings:

1. From the observed nmr line broadening of the methyl protons of 3-methylsalicylate (I) in solution of horse heart ferricytochrome c (ref. 1970 report #1), (I) is found to be bound to the latter with the methyl group at 4∿6 Å from the heme iron. The uncertainty arises mainly from the electron spin relaxation time assumed. Analysis of the epr measurements on the same system indicates that the crystal field around the heme iron has a stronger axial as well as rhombic distortion as the result of the binding of (I). The most probable binding site would be in one of the two hydrophobic "channels" leading to the heme group.

2. (With Dr. Hirasawa)
Epr studies of the single crystal of histidine hydrochloride doped with a few mol per cent of Cu^{2+} showed that the environment of the Cu^{2+} ion is entirely different than in the normal 1:2 Cu^{2+}-histidine complex molecule. This is best illustrated by the unique situation in this doped system that the unpaired electron has to be assigned to the axial d-orbital of Cu^{2+} ion rather than the in-plane orbitals as in most of the systems studied before. The proposed site of Cu^{2+} in the known structure of histidine HCl single crystal can consistently explain the optical and epr results, and indeed confirms in detail the proposed differences in behavior by the Cu^{2+} ion relative to the histidine environment.

Significance to NIAMD Research:

These investigations contribute basic information toward understanding interactions involving metal ions, small organic molecules and the protein in more complicated and biologically significant systems.

Proposed Course of Project:

The type of investigation outlined above will be continued. In addition, some modifications of our present epr system is being conducted (with Dr. Hirasawa) so that some fast phenomena involving a paramagnetic protein and other systems can be studied by a rapid scanning epr method.

Publications:

Gotto, A. M. and Kon, H.: Observations on the conformation of human serum high-density lipoproteins using infrared spectroscopy, circular dichroism and electron spin resonance. Biochem. 9: 4276-4282, 1970.

Makinen, M. W. and Kon, H.: Circular dichroism and electron paramagnetic resonance of the haptoglobin-hemoglobin complex. Biochem. 10: 43-52, 1971.

Miller, J. H., White, F. H., Jr., Riesz, P. and Kon, H.: Distributions of free radicals among amino acids from lyophilized ultraviolet-irradiated proteins. Photochem. Photobiol. In press.

REPORTED IN PRESS - 1970

Lousberg, R. J. J. Ch., Paolillo, L., Kon, H., Weiss, U., and Salemink, C. A.: Pigments of Elsinoe species. Part IV. Confirmatory evidence for the structure of Elsinochrome A and its ethers from studies of nuclear magnetic resonance (solvent and Overhauser effects) and electron spin resonance. J. Chem. Soc. (C): 2154-2159, 1970.

White, F. H., Jr., Kon, H., and Piesz, P.: Comparison of electrical discharge with γ-irradiation for the production of free radicals in lyophilized proteins. Rad. Res. 45: 8-24, 1971.

Serial No. NIAMD-LPB-13
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Molecular Dynamics

Previous Serial Number: NIAMD-LPB-14

Principle Investigator: Ira W. Levin

Other Investigators: William C. Harris

Cooperating Units: S. Abramowitz, National Bureau of Standards
T. Lewis, NIAMD-LPB and City College, New York
C. Berney, University of New Hampshire, Durham, N.H.
O. W. Adams, National Science Foundation
K. Wiberg, Yale University, New Haven, Conn.

Man Years

Total:	1.5
Professional:	1.5
Other:	0

Project Description:

Objectives:

An understanding of the vibrational characteristics of polyatomic systems through the analysis of Raman and infrared spectra, the determination of reliable intramolecular force fields and the interpretation of molecular charge distribution (intensity) parameters.

Methods Employed:

Various laser Raman and infrared spectroscopic procedures provided the required data for the vibrational analyses. Both the intramolecular force field determinations and large molecular orbital calculations were necessarily performed on the digital computer.

Major Findings:

1. A complete vibrational assignment for bicyclo (1.1.1) pentane, relatively high symmetry (D_{3h}) and large strain energy (about 50 kcal/mole), and for the $-d_1$ and $-d_2$ isotopes was made on the basis of the infrared gas-phase and Raman liquid data. The descriptions of the

normal modes, based upon normal coordinate analyses, presents a means toward understanding the vibrational coupling mechanisms characteristic of rigid ring systems. (Levin, Lewis and Wiberg)

2. The in-phase and out-of-phase methyl torsional modes in the Raman spectrum of polycrystalline [$(CH_3)_2C{=}CH_2$] were observed for the first time. The spectral interpretation was verified by an examination of the torsional spectra of isobutene-d_6 and isobutene-d_8. The spectral data give a torsional barrier of 2.8 kcal/mole. The increase in barrier height over single methyl group systems probably arises from methyl-methyl interactions. (Levin and Harris)

3. We explored the use of Raman spectra obtained at low temperatures for resolving complicated spectral patterns and for eliminating assignment ambiguities in molecular systems containing two methyl groups bonded to a common atom. The fundamental modes of isobutene-d_0, isobutene-d_6 and isobutene-d_8 were assigned on the basis of group frequency correlations, isotopic shift ratios and previously reported infrared band contours. The vibrational assignment was discussed in conjunction with existing intramolecular force fields. (Levin and Harris)

4. The integrated infrared intensities of the fundamental vibrations of PF_3 were experimentally determined using nitrogen broadening pressures of 550 psi. With CNDO-2 molecular orbital calculations as a guide, the redistribution of the electronic charge during the fundamental vibrations was discussed as a function of net atomic charge density and atomic polarization terms. In an effort to sort out effects arising from d electrons and unshared electron pairs, the charge reorganizations for PF_3 were compared to those for NF_3. (Levin and Adams, O.W.)

5. We explored a novel method of a priori calculating an infrared spectrum (frequencies and intensities) for a molecule using approximate molecular orbital techniques in conjunction with normal coordinate analyses. SF_4 represented a test system as several sets of vibrational assignments have been suggested. Comparison of the calculated spectra with the observed spectrum allowed one to differentiate between the various sets of proposed assignments. (Levin and Berney)

6. For systems in which identical nuclei occupy nonequivalent sites, exchange between sites can occur through pseudorotation. No experimental vibrational evidence exists for the effect and only estimates for the tunneling frequencies and barriers appear in the literature. SF_4 exhibits a series of hot band transitions in the far infrared which we believe arise from pseudorotation. We interpret the spectral series in terms of a double minimum potential function that would be characteristic of pseudorotation. The interpretation leads to a barrier height of 10.1 kcal/mole. (Levin and Harris)

Significance to NIAMD Research:

Since experimentally determined vibrational frequencies and charge distribution parameters reflect the sum of several subtle electronic and nuclear contributions, force field and molecular orbital calculations permit both quantitative and qualitative descriptions of the dominant terms and create a link between empirical information and bond properties.

Proposed Course of Project:

We are continuing studies on highly strained bicyclic ring systems; namely, norbornane, norbornadiene and quadricyclene, with strain energies of 17.5, 34.7 and 101.1 kcal/mole, respectively. Specifically we plan to assign in detail the vibrational spectra and to examine modified valence force fields for these systems. In order to understand the charge redistributions that arise in strained molecules, we will investigate, experimentally and theoretically, the intensity parameters of bicyclobutane, a highly strained system (66.5 kcal/mole) which is particularly amenable to molecular orbital techniques.

Experimentally, a strong emphasis will be placed on development of laser Raman matrix techniques. Application of Raman intensities to structural problems will also be initiated.

Publications:

Harris, W. C., and Levin, I. W.: Direct observation of the torsional vibrations in isobutylene, isobutylene-d_6 and isobutylene-d_8 by Raman scattering. J. Chem. Phys. In press.

Lewis, T. P. and Levin, I. W.: Absolute infrared intensities of hydrocarbons, Theoret. Chim. Acta (Berl.) 19: 55-65, 1970.

Abramowitz, S. and Levin, I. W.: Raman spectra of the hexahalogenated benzenes in the solid phase. Spectrochim. Acta 26A: 2261-2268, 1970.

Levin, I. W. and Abramowitz, S.: Isotopic splitting in matrix isolated BF_3. Chemical Physics Letters 8. In press.

REPORTED IN PRESS - 1970

Levin, I. W., Milne, G. W. A. and Axenrod, T.: Raman and infrared spectra of dimethylnitrosamine. J. Chem. Phys. 53: 2505-2512, 1970.

Serial No. NIAMD-LPB-14
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Electronic and molecular structural investigations

Previous Serial Number: NIAMD-LPB-15

Principal Investigator: Ruth McDiarmid

Other Investigators: None

Cooperating Units: None

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To describe the electronic and molecular structures of ground and excited states of simple molecules.

Methods Employed:

The measurement and analysis of electronic absorption spectra, predominantly in the ultraviolet. The calculation, with digital computers, of energy levels and geometries to correlate with and further explain the experimental observation.

Major Findings:

1. The vibrational structure of the lowest electronic transition of ReF_6 has been analyzed. Four of the six fundamental frequencies have been shown to retain their ground state values. One could not be observed. The sixth fundamental has been shown to have a very different frequency than is observed in the ground electronic state. From this, and other data, the ground state fundamental frequency was concluded to be perturbed, as had been previously postulated.

2. The higher energy spectrum of ReF_6 was shown to contain three other electronic transitions. Two of these are members of a Rydberg series leading to an ionization potential of 7.99 eV. The third can not be rigorously assigned.

3. The vibrational structure of the 1795 Å transition of isobutene and three of its partially deuterated isomers were analyzed. A comparison of the upper and ground state vibrational frequencies was made, from which it was concluded that the electronic structure of the molecule had undergone an appreciable alteration upon excitation. An analysis of the methyl torsional frequencies revealed that the molecular structure of the molecule had also changed.

4. The 1795 Å transition of isobutene was shown to arise from a $\sigma \leftrightarrow \pi$ transition, but whether this is 3pRyd $(\sigma^*) \leftarrow \pi$ or $\pi^* \leftarrow \sigma$ can not be unambigiously concluded.

Significance to NIAMD Research:

This research project seeks to understand the electronic changes a molecule undergoes on absorbing light and, more significantly, the structural alterations that a given electronic change can cause. This study can, if fruitful, provide a basis for a physical interpretation of the relative spectroscopic results obtained in other, more general studies.

Proposed Course of Project:

1. The analysis of the spectrum of 2-methylpropene (isobutene) will be continued with the goal of identifying and characterizing its higher energy transitions.

2. The spectroscopic study of the V←N transition of ethylene continues. It is hoped that through an examination of (1) partially deuterated ethylenes and (2) low intensity bands of tetradeuteroethylene the remaining problems can be resolved.

3. Specifically fluorinated ethylenes will be examined spectroscopically to determine the effect of substitution on the unperturbed ethylene spectrum.

4. Other metal hexafluorides will be studied to (1) locate their Rydberg transitions and (2) help in the analysis of ReF_6.

Publications:

McDiarmid, R.: Calculation of the energies of the lower excited states of CH_3. Theoret. Chim. Acta 20: 282-291, 1971.

McDiarmid, R.: Jahn-Teller effects in the $^2E_{5/2g} \leftarrow {}^2G_{3/2g}$ transition of ReF_6. J. Mol. Spectry. In press.

McDiarmid, R.: Higher electronic states of ReF_6. J. Mol. Spectry. In press.

Serial No. NIAMD-LPB-15
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanisms of energy transfer at cellular sites of photochemical action.

Previous Serial Number: NIAMD-LPB-16

Principal Investigator: Dr. Rodney A. Olson

Other Investigators: None

Cooperating Units: None

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To interpret the mechanisms of energy transfer and allied metabolic steps at cytological sites of photochemical action. To determine the role of chromophore orientation in laminar photoreceptors (chloroplasts, etc.) and in crystalline pigment-protein systems and paracrystalline pigment-lipid systems simulating the organized structure of the photosynthetic apparatus.

Methods Employed:

Methods include those required for the study of absorption, emission, excitation and polarization spectra in areas of living cells and microcrystals in the range one to ten square microns. Currently this study has been extended into the realm of cryogenic microspectrophotometry wherein the resultant sharpening of spectra permits the resolution and measurement of polarization spectra unobscured by "vibrational overlap" caused at normal temperature by components absorbing or emitting in adjacent spectral regions. Specimens are positioned in a 'cold finger' assembly attached to the bottom of the liquid nitrogen well of a metal cryostat. A windowed vacuum shroud surrounds the cold finger assembly, and the intervening space (continuous with the void of the cryostat) is pumped down to 10^{-6} torr. The specimen aqueous environment is isolated from the vacuum system by a pair of small windows separated by appressed indium gaskets.

The microscope optics employed require long working distances and high magnification. A pair of custom made reflecting objectives meet these requirements (working distance = 19.0 mm, magnification = 150 X). These are incorporated in a massive horizontal optical bench with appropriate modular elements including high resolution monochromators and chilled infrared sensitive photomultipliers. The multiplicity of measurements required by the spectral fine structure at low temperature is provided conveniently by digital timing and control circuitry whereby appropriate sample-and-hold procedures and operational amplifiers permit direct recording of the wavelength dependence of both polarization spectra and dichroic ratio.

Major Findings:

Experimental evaluation of the optical system developed in this laboratory for the low temperature (77°K) study of the polarization spectra (dichroism, bifluorescence, etc.) of photoreceptor organelles and microcrystals of pigment-protein complexes indicates a promising new approach to the determination of the molecular orientation and structural arrangement of the chromophores involved in these structures.

The resultant sharpening of both absorption and emission spectra at 77° permits spectral identification of oriented species of chromophores. In Bacteriochlorophyll protein crystals, for example, the absorption peak at 809 nm and its adjacent shoulder is resolved into three distinct peaks and a shoulder while in Euglena chloroplasts four distinct emission species of chlorophyll can be resolved in place of the broad peak and shoulder normally observed at room temperature. The degree and direction of the polarization of these spectral components can be determined with the low temperature apparatus here described. Detailed measurements of these values await the refinement and final modification of the pilot system evaluated here.

Significance to NIAMD Research:

Since the fine structure of photoreceptors and pigment-protein complexes may be closely related to their role in photochemical processes polarization studies may lead to a better interpretation of energy transfer in all living cells, whether activated chemically or by light.

Proposed Course of the Project:

Continuation of the cryogenic polarization spectral measurements described above in the direction of resolving by fluorescence excitation spectra, energy trapping sites and the effects of bound electron donors and acceptors.

Publications:

Labaw, L. W. and Olson, R. A.: Further electron microscopic observations of bacteriochlorophyll protein crystals. J. Ultrastructural Res., 31: 456-464, 1970.

Olson, R. A.: Microtubular spherulites: Development and growth in solutions of bacteriochlorophyll protein. Science, 169: 81-82, 1970.

Serial No. NIAMD-LPB-16
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Spectral, physical-chemical and photochemical properties of biologically active substances

Previous Serial Number: NIAMD-LPB-17

Principal Investigator: Norman E. Sharpless

Other Investigators: None

Cooperating Units: Frederick S. Brackett, Consultant, LPB-NIAMD
Charles L. Greenblatt, Hadassah Medical School, The Hebrew University, Jerusalem, Israel
Ralph G. Adams (LPB-NIAMD)
William Jennings (LPB-NIAMD)
Robert B. Bradley (LPB-NIAMD)
James A. Ferretti (CR-PSL)

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

The investigation of the influence of molecular structure on physical and biological properties, with the object of correlating physical and chemical properties with biological activity.

Methods Employed:

These investigations utilize various forms of spectrophotometry (visible, UV, IR, NMR and ERS), which distinguish energy levels; ORD, CD and gas chromatography, which distinguish other molecular properties; and theoretical calculations of quantum chemical values.

Major Findings:

Photochemistry of the Vitamin D System (with Dr. Brackett)

A continuation of the mathematical analysis of this system indicates that a previously unknown intermediate, similar spectroscopically to tachysterol but of much greater stability, occurs in the system.

Retinaldehyde-Phospholipid Interactions (with Dr. Adams and Mr. Jennings)

The chromophoric portion of bovine-rhodopsin is generally acknowledged to (1) contain the 11-cis isomer of retinaldehyde, (2) to be highly photolabile and (3) to have an absorption maximum near 500 nm. The complexing of Ethanolamine phosphoglyceride with various analogues of retinaldehyde has been examined. It has been found that the methyl groups of the retinaldehyde play a significant role, and the electron density (q) at various centers in the chain is one factor controlled by these methyl groups, as evidenced by changing q's in the chain; investigated by synthesizing compounds in which a C=C group has been replaced by a C=N group.

The extremely complicated kinetic equations of this system (a reversible second order reaction followed by two sequential reversible first order reactions: A + B C + D; C D E) is undergoing active investigation. Preliminary computer programs have been examined, and orders of magnitude for the reaction rate constants estimated.

Nuclear Magnetic Resonance Spectroscopy of Heterocyclic Fused Ring Compounds (with Mr. Bradley and Dr. Ferretti)

Some compounds with identical heterocenters have been analyzed, and their spectra shown to have normal AA'BB' spectra, as anticipated. However, those compounds with non-identical heterocenters (as exemplified by xanthone) were found not to possess the anticipated ABCD spectra but to have, rather, an XAMK type, in what would have been considered a priori, a reverse order.

Therapeutic Interference (with Dr. Greenblatt)

The spin label required for these investigations has been synthesized.

A paper on interference competition "Therapeutic Interference Studies. Mechanisms of Interpolymer Competition for Dyes" by Sharpless, Greenblatt, and Jennings has been submitted to Molecular Pharmacology.

Significance to NIAMD Research:

The proper understanding of the behavior of biologically active substances at the molecular level is necessary for the understanding of their actions at the cellular level in order to evaluate their contributions to both health and diseased states.

Proposed Course of Project:

Therapeutic interference studies will be continued utilizing 1) spin labeling and 2) other dyes with lower symmetry than Crystal Violet (i.e., Malachite Green) to a) investigate the influence of symmetry properties on binding and interference and b)(of a practical nature) to separate spectrophotometric absorption bands for easier analysis.

Studies will be carried out on the kinetic analysis of the EPG-retinaldehyde reaction to establish the mechanism of the reaction.

Publications:

Greenblatt, C. L., Sharpless, N. E., and Yamaoka, K.: Competition for polymeric binding sites between acridine and triphenylmethane dyes. Mol. Pharmacol. 6:649-658 (1970).

Sharpless, N. E., Adams, R. J., and Jennings, W. H.: A molecular model for a phospholipid-retinaldehyde complex absorbing at 500 NM. Photochem. Photobiol. 13:21-26 (1971).

Ferris, J. P., Boyce, C. B., Briner, R. C., Weiss, U., Qureshi, I. H., and Sharpless, N. E.. Lythraceae alkaloids X. Assignment of absolute stereochemistries on the basis of chiraloptical effects. J. Am. Chem. Soc. 93: in press (1971).

Serial No. NIAMD-LPB-17
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Chemistry and biosynthesis of natural compounds, and instrumental methods used in their study.

Previous Serial Number: NIAMD-LPB-18

Principal Investigator: Ulrich Weiss

Other Investigators: Karl H. Weisgraber
Izhar H. Qureshi

Cooperating Units: Prof. C. A. Salemink, University, Utrecht, The Netherlands
Dr. R. J. J. Ch. Lousberg, University, Utrecht, The Netherlands
Dr. Keith S. Brown, Jr., Centro de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil
Dr. J. M. Edwards, School of Pharmacy, University of Connecticut, Storrs
Dr. S. Liaaen Jensen, Department of Chemistry, Norwegian Polytechnic Institute, Trondheim, Norway
Dr. Isabella L. Karle, Naval Research Laboratory
Dr. James V. Silverton (NHI-LC)

Man Years

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

Isolation in pure form, and elucidation of the chemical constitution of a variety of naturally occurring substances of chemical, biosynthetic, or biological interest. Study of instrumental techniques used in such work. Corroboration of chemical structure by total synthesis.

Methods Employed:

Standard procedures or organic chemistry, and instrumental methods (UV, IR, NMR spectroscopy). For measurement of optical rotatory dispersion and circular dichroism, the Cary 60 spectropolarimeter and its CD attachment are used.

Major Findings:

Earlier work on the CD of heteroannular cisoid dienes has been resumed; their chiraloptical effects are exceptional in showing a sign opposite to the one predicted from our otherwise well-established "diene rules." Theories to account for this anomaly have been examined by Dr. Charney. For details, see the report by Dr. Ziffer.

The CD of morphine derivatives has been re-investigated by Dr. Qureshi with modern instrumentation. The results extend earlier (1964) findings made with less sophisticated equipment, and confirm the role of the slightly non-planar aromatic chromophore of morphine in causing the observed CD effects. The unusual conformation of levopimaric acid, deduced in 1961 from our ORD measurements, has been fully confirmed by x-ray crystallographic work by Dr. I. L. Karle.

Attempts at total synthesis of the mold pigment, elsinochrome A, have led to a surprisingly simple method of synthesis of a related perylene-quinone. The synthetic approach, for which a precedent was found only after completion of the work, may be generally useful for synthesis of polycyclic compounds.

In cooperation with Dr. Edwards, the unusual perinaphthenone pigments of the plant, Lachnanthes tinctoria, have been studied further. The flowers yield two new pigments, representing a new type but related to those of roots and seed-capsules. The unique biosynthesis of plant perinaphthenones from one molecule each of phenylalanine, tyrosine, and acetic acid, postulated by Dr. R. Thomas for such a pigment from an Australian plant related to ours, has been confirmed for one of the root pigments of Lachnanthes; it has been shown that the one carbon atom lost during biosynthesis of the ring system from the three precursors is the carboxyl of the acetic acid.

In cooperation with Dr. Ziffer our earlier observations on photochemical transformations of 9-bromophenanthrene have been clarified by Dr. Edwards. For details, see the report by Dr. Ziffer.

In continuing study with Dr. K. S. Brown of the pigments of the bright orange aphid, Aphis nerii, two strongly red-fluorescing constituents of very unusual chemical structure, present in very low concentration, have been studied. Together with Dr. Jensen, the yellow carotenoids of the green form of the aphid Macrosiphum liriodendri, isolated pure by Dr. Weisgraber, have been found to be the first representatives of this class containing γ-ionone rings. One of these carotenoids has been isolated independently by Dr. Jensen from a discomycete fungus. For a crystalline metabolite of the mold, Corticium caeruleum, isolated pure in very small amounts by Dr. Weisgraber, nmr evidence suggests an unprecedented structure and uncommon biosynthesis. Because only minute amounts are available, the exact structure of the compound is being studied by Dr. Silverton with the help of x-ray crystallography.

Proposed Course of Project:

It is hoped to complete the study of aphid pigments (Aphis nerii, Macrosiphum liriodendri) by collection of additional batches when the insects are available in April and May. Study of the red-fluorescing trace constituents of the former, and detailed investigation of the distribution of yellow and pink carotenoids in green and pink forms, respectively, of the latter are required, as is study of some of the very sensitive pink carotenoids.

Work towards the synthesis of elsinochrome, and on the blue mold pigments (see 1969/1970 report) is to be resumed, if possible. The x-ray crystallographic structure analysis of the colorless metabolite from Corticium should be completed soon.

Publications:

Brown, K. S., Jr., and Weiss, U.: Chemical constituents of the bright orange aphid, Aphis nerii Fonscolombe. II. Structure of some minor constituents. Anais Acad. Brasil Ciências. In press.

Weiss, U., Edwards, J. M., and Ziffer, H.: Some photochemical reactions of 9-bromophenanthrene. Austral. J. Chem. In press.

Ferris, J. P., Boyce, C. B., Briner, R. C., Weiss, U., Qureshi, I. H., and Sharpless, N. E.: Lythraceae alkaloids. X. Assignment of absolute stereochemistries on the basis of chiraloptical effects. J. Amer. Chem. Soc. In press.

REPORTED IN PRESS - 1970

Lousberg, R. J. J. Ch., Weiss, U., and Salemink, C. A.: Pigments of Elsinoe species. Part III. Methylation of Elsinochrome A. Formation of mono- and dimethyl-ethers. J. Chem. Soc. (C): 2152-2154, 1970.

Lousberg, R. J. J. Ch., Paolillo, L., Kon, H., Weiss, U., and Salemink, C. A.: Pigments of Elsinoe species. Part IV. Confirmatory evidence for the structure of Elsinochrome A and its ethers from studies of nuclear magnetic resonance (solvent and Overhauser effects) and electron spin resonance. J. Chem. Soc. (C): 2154-2159, 1970.

Lousberg, R. J. J. Ch., Salemink, C. A., and Weiss, U.: Pigments of Elsinoe species. Part V. The structure of Elsinochrome D. J. Chem. Soc. (C): 2159-2162, 1970.

Edwards, J. M., and Weiss, U.: Perinaphthenone pigments from the fruit capsules of Lachnanthes tinctoria. Phytochemistry 9: 1653-1657, 1970.

Edwards, J. M., Churchill, J. A., and Weiss, U.: A chemical contribution to the taxonomic status of Lophiola americana. Phytochemistry 9: 1563-1564, 1970.

Edwards, J. M., and Weiss, U.: Oxidation products of 2,3,6,7-tetramethoxy-9,10-dimethylphenanthrene. Can. J. Chem. 48: 1785-1787, 1970.

Serial No. NIAMD-LPB-18
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Titles:

1. A study of photoreactions and the mechanisms by which these reactions occur.
2. A study of the interdependence of CD (or ORD) curves and the structure and stereochemistry of dienes.
3. A study of the induced CD bands in optically inactive compounds by optically active solvents.

Previous Serial Number: NIAMD-LPB-19

Principal Investigator: Herman Ziffer

Other Investigators: None

Cooperating Units: Donald M. Jerina (NIAMD-LC)
J. M. Edwards, University of Connecticut
U. Weiss (NIAMD-LPB)
E. Charney (NIAMD-LPB)
E. Bunnenberg, Stanford University

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

1. An examination of the optical activity of a number of heteroannular cisoid dienes and a comparison of the sign and magnitude to that predicted by theory.
2. To determine the absolute stereochemistry of (+)-cis-2,3-dihydroxy-1-methyl cyclohexa-4,6-diene, a microbial metabolite of toluene. To compare similar metabolites of the same enzyme system in an effort to learn more about the enzyme-substrate complex.
3. An examination of the photochemistry of 9-bromophenanthrene and a comparison of its photochemistry with that of other halogenated polycylic hydrocarbons.
4. To study the CD bands of substituted benzophenones induced by optically active solvents.

5. To learn more about the photoreactivity of β,γ-unsaturated ketones.

Methods Employed:

The Cary 60 spectropolarimeter has been used to obtain ORD and CD measurements. Standard organic chemical techniques were employed for the synthesis and proof of structure of a number of compounds. Along with the usual spectroscopic measurements (NMR, IR and UV) we have used the Varian time averaging computer in conjunction with our NMR equipment to obtain several important NMR measurements necessary for structure determinations.

Major Findings:

1. The optical activity of several heteroannular cisoid dienes was measured and found to be opposite in sign to that predicted by the diene rule. These results have been analyzed in terms of more sophisticated treatments of the optical activity of dienes.
2. Several of the four possible 3-methyl-1,2-cyclohexane-diols have been synthesized and their stereochemistry determined. The hydrogenation product of (+)-cis-2,3-dihydrozy-1-methylcyclohexane has been shown to be identical to one of the diols. The relative stereochemistry of the methyl and hydroxyl groups has been determined as trans.
3. The structure of a photoproduct produced in the irradiation of a benzene solution of 9-bromophenanthrene has been determined and confirmed by synthesis.
4. The CD bands induced in a series of substituted benzophenones by an optically active solvent have been found to be remarkably similar in sign and magnitude. A mechanism for the induction of these bands has been suggested.

Significance to NIAMD Research:

Information on the absolute stereochemistry of metabolites will assist in developing models which describe the way substrates attach to enzymes and the course of the reaction.

The increased knowledge derived from studies of the mode and mechanism of photochemical reactions permit the synthesis of compounds that are difficult to prepare by other means.

Proposed Course of Project:

1. To determine the absolute stereochemistry of (+)-cis-2,3-dihydroxy-1-methylcyclohexa-4,6-diene as well as that of several related dihydrodiols obtained as microbial metabolites.
2. To continue the study of the relationship between optical activity to structure and stereochemistry of dienes.
3. To continue the study of the photochemical reactivity of β,γ-unsaturated ketones and several other chromophores.

Publications:

Ziffer, H.: Some photochemical reactions of 9-bromophenanthrene. Australian J. of Chem., in press.

Serial No. NIAMD-LPB- 19
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of altitude (hypoxia), exercise, and other environmental stresses on physiological, biochemical and pathological mechanisms in animals.

Previous Serial Number: NIAMD-LPB-20

Principal Investigator: Paul D. Altland

Other Investigators: Mr. H. F. Brubach and Dr. M. P. Dieter

Cooperating Units: Dr. Benjamin Highman, Section on Pathologic Anatomy, Laboratory of Experimental Pathology, NIAMD, LEP-2 A.

Man Years (July 1970 through June 1971)

Total:	3.5
Professional:	1.5
Other:	2

Project Description:

Objectives:

To determine physiological, biochemical, and pathological changes during exposure to altitude, strenuous exercise and cold, and to study the mechanisms of action of cross adaptation between such stresses. To determine effects of altitude acclimatization on exercise performance at sea level. This requires evaluation of effects of polycythemia on exercise performance. To determine the effects of cold acclimatization on capacity of animals to exercise in the cold or at room temperature and to determine the effects of exposure to cold on tissue and enzyme changes. To study physiological and biochemical effects of smoke inhalation on exercise performance in rats.

Methods Employed:

Biochemical analyses were made of serum and tissue enzymes and metabolic end products. Physiological observations were made of respiration and body temperature. Histopathological methods were used for demonstration of fatty and necrotic changes in vital organs. Two altitude chambers, an animal exerciser, a cold chamber and a gas chamber modified for introduction of tobacco and corn silk smoke were used.

Major Findings:

Exposure of rats to an atmosphere of smoke from tobacco (nicotone < 1.8 mg, tar < 2.0 mg/cigarette) did not affect survival in 20.9 or 11.8% O_2 (15,000 ft. equivalent). At lower O_2 concentrations the mortality of rats was greater in tobacco smoke than in corn silk smoke, which was attributed to higher COHb saturations (corn silk 36%, tobacco 44%) in the tobacco smoke. Rats exposed to 0.045% CO in 8.3% O_2 (23,000 ft equivalent) without smoke had a higher survival than rats exposed to tobacco smoke in 8.3% O_2. Both types of smoke produced an immediate reduction in breathing rate in all O_2 concentrations from 20.9 to 6.6% O_2 (28,000 ft equivalent), but .045% CO alone had no effect. Hypothermia produced in rats by hypoxia was intensified only by exposure to tobacco smoke. Blood glucose and serum lactic acid values were higher in rats in tobacco smoke than in corn silk smoke at all hypoxic levels. The critical O_2 concentration which produced "excess lactate," an index of anaerobic metabolism, was 6.6% O_2 without smoke, 8.3% O_2 in corn silk smoke, and 10.4% in tobacco smoke.

Cold acclimated rats exercised for 3, 5, and 9 hr in a cold room (1.7 C) survived, but two-thirds of a group of unacclimated rats died during 9 hrs exposure. Exposure to cold induced a significant rise in serum aldolase (SAld) and serum lactic dehydrogenase (SLDH) activity. After cold acclimation serum glutamic oxalacetic transaminase (SGOT) and SLDH were significantly greater than controls. After exercise in the cold the rise in serum enzyme activity was greater in unacclimated rats. A similar response was observed in tissue enzymes.

Significance to NIAMD Research:

Our work clearly shows that anaerobic metabolism is greatly accelerated in rats in an atmosphere of tobacco smoke and that tobacco smoke produces a marked hypothermia in animals in a low O_2 atmosphere.

It has generally been accepted that cold acclimation of animals occurs within 4 weeks. The presence of an excess of certain enzymes in the serum after 4 weeks exposure to cold suggests that cold acclimation is incomplete at this time. These results also suggest that there is a degree of positive cross acclimation between cold and exercise, despite earlier findings that cold acclimatized rats exercise poorly at room temperature.

Proposed Course of Project:

The importance of polycythemia on the capacity of rats to exercise and the effect of an atmosphere of smoke on the capacity of rats to exercise and upon biochemical changes in the serum will be studied. Emphasis will be placed on differentiating the effects of carbon monoxide and nicotine. Studies will also be conducted on the effects of exposure of rabbits to cold on the changes induced in tissue and in serum enzymes.

Publications:

Dieter, M. P., Altland, P. D., and Highman, B.: Tolerance of unacclimated and cold-acclimated rats to exercise in the cold. Serum, red and white muscle enzymes, and histological changes. Canad. J. Physiol. and Pharmacol. 48: 723-731, Oct. 1970.

Altland, P. D., Brubach, H. F., Parker, M. G., Dieter, M. P., and Murayama, M.: Effects of smoke on tolerance of rats to hypoxia. J. Appl. Physiol. (in press).

Altland, P. D., and Highman, B. (with technical assistance of M. G. Parker): Effects of polycythemia and altitude hypoxia on rat heart and exercise tolerance. Am. J. Physiol. (in press).

REPORTED IN PRESS - 1970

Altland, P. D., Highman, B., Parker, M. G., and Dieter, M. P.: Serum enzyme, corticosterone and tissue changes in rats following a single oral dose of ethanol. Quart. J. Studies Alcohol 31: 281-287, June 1970.

Serial No. NIAMD-LPB-20
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effect of environmental stresses on cellular homeostasis.

Previous Serial Number: NIAMD-LPB-21

Principal Investigator: Michael P. Dieter

Other Investigators: Paul D. Altland and Benjamin Highman

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To make a comparative study of the biochemical mechanisms responsible for the control of cellular homeostasis.

Methods Employed:

Spectrophotometric assay of enzymes and substrates, fluorometric assay of hormones, colorimetric assay of reduced and oxidized vitamin C, preparatory ultracentrifugation.

Major Findings:

Bioassays for the activity of cell-free thymic extracts (thymic humoral factor or THF) were performed using preparations from guinea pigs maintained six weeks on four known levels of vitamin C intake, or on dialyzed extracts of rat thymus from which vitamin C had been partially removed. It was found that the concentration of dehydroascorbate in the guinea pig thymuses increased, and that of ascorbate (and total vitamin C) decreased in direct proportion to the level of vitamin C in the diet. The activities of the various THF preparations were found to be directly correlated with the level of ascorbate and inversely correlated with the level of dehydroascorbate in them. The data indicated that throughout ontogeny of the thymus the level, and

particularly the oxidation state of thymic vitamin C, was involved in some manner for the production of THF. No evidence was found that vitamin C was required for the actual expression of THF activity, since prior to bioassay, the partial removal of ascorbic acid from similarly prepared rat THF preparations did not result in diminished responses.

Injection of immature rats and cockerels with graded doses of testosterone or corticosterone decreased the concentration of total vitamin C (ascorbate and dehydroascorbate) in the lymphoid organs, independent of any hormonal influence on vitamin C biosynthesis, with the percentage of dehydroascorbate increasing and that of ascorbate decreasing. The largest per cent change occurred in the central lymphoid organs, the bursa and thymus, rather than in the peripheral lymphoid organs, the spleen and lymph nodes. The data suggests that the ascorbate-dehydroascorbate system in lymphoid organs may be an integral portion of the physiological mechanisms operative during hormonal modulation of the growth, metabolism and function of lymphoid organs.

Significance to NIAMD Research:

Elucidation of the nature and function of the hormone-like thymus factor contributes to our understanding of mammalian immunobiology. Since production of this factor by the thymus can be diminished by lowering vitamin C intake in guinea pigs, and the factor is necessary for normal cell-mediated immune responses, alteration of the latter by steroid hormone treatment and/or vitamin intake may be feasible in primates.

Proposed Course of Project:

1. The interrelationship of vitamin C and thymic humoral factor to certain of the cell-mediated immune responses in guinea pigs are being investigated.

2. The extent of participation of bone marrow as a component necessary for the expression of thymic humoral factor activity is under investigation.

Publications:

Dieter, M. P., Altland, P. D. , and Highman, B.: Tolerance of unacclimated and cold-acclimated rats to exercise in the cold: Serum, red, and white muscle enzymes and histological changes. Canad. J. Physiol. Pharmacol. 48: 723-731, 1970.

Dieter, M. P.: Further studies on the relationship between vitamin C and thymic humoral factor. Proc. Soc. Exptl. Biol. Med. 136: 316-322, 1971.

Altland, P. D., Brubach, H. F., Parker, M. G., Dieter, M. P., and Murayama, M.: The effects of smoke on tolerance of rats to hypoxia. J. Appl. Physiol., (in press).

Dieter, M. P., and Breitenbach, R. P.: Vitamin C in lymphoid organs of rats and cockerels treated with corticosterone or testosterone. Proc. Soc. Exptl. Biol. Med., (in press).

REPORTED IN PRESS - 1970

Altland, P. D., Highman, B., Parker, M. G., and Dieter, M. P.: Serum enzymes, corticosterone, and tissue changes in rats following a single dose of ethanol. Quart. J. Studies on Alcohol 31: 281-287, 1970.

Serial No. NIAMD-LPB-21
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Physico-chemical properties of biological surfaces and related systems.

Previous Serial Number: NIAMD-LPB-22

Principal Investigator: N. L. Gershfeld

Other Investigators: R. E. Pagano

Cooperating Units: None

Man Years (July 1970 through June 1971)

Total:	1-1/4
Professional:	1-1/4
Other:	0

Project Description:

Objectives:

(a) To measure the intermolecular energies of condensed monomolecular lipid films.

(b) to evaluate the influence of lipid structure on the properties of cell surfaces.

Methods Employed:

General techniques of surface chemistry have been adapted and refined to measure intermolecular energies at fluid interfaces.

Major Findings:

Measurement of the internal energy of condensed lipid films, Ei, indicates that the lipid region is energetically equivalent to bulk hydrocarbon liquids. Values for Ei are given in Table I for the

liquid-expanded and liquid-condensed monolayer states; these two physical states encompass most of the naturally occurring lipids which form monolayers on water. Included in Table I for comparison are literature values for bulk solid and liquid hydrocarbons.

Table I

Physical State of Hydrocarbon	Ei Kcal/mole CH_2	
Bulk		
Solid	2.3	
liquid	1.4	
Monolayer		
liquid-condensed	1.6	(saturated aliphatic compounds)
liquid-expanded	1.3	(unsaturated aliphated compounds)

The contribution of the polar groups to the internal energy of the condensed lipid films is significant and the results suggest varying degrees of influence on the adjacent aqueous phase; both ordering and disordering of the water structure is observed. These effects depend on the nature of the polar group as well as the physical state of the lipid film..

Lipid mixtures in surfaces are dominated by interaction in the hydrocarbon domain of the films. The addition of cholesterol to a homogeneous lipid film (e.g. Lecithin) can lead to either large positive deviations from ideal (i.e. zero heat) mixing or to phase separation.

Significance to NIAMD Research

Cholesterol in lipid structures appears to maintain the aggregate in a state which is poised to undergo a phase transition. This is in direct opposition to the generally accepted view that cholesterol stabilizes the structure of lipid aggregates. The significance of this finding is that cholesterol may be acting as part of a control system which mediates phase changes in the lipid aggregates in membranes.

Proposed Course of the Project

The influence of cholesterol on the structure of lipids in membranes of mycoplasma is presently being studied.

Publications:

Gershfeld, N. L., Pagano, R.E., Friauf, W. S., and Fuhrer, J. Millidyne Sensor for the Langmuir Film Balance. Rev. Sci. Instr. 41, 1356-1358, 1970.

Serial No. NIAMD-LPB-22
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of metabolic activity in microorganisms.

Previous Serial Number: 23

Principal Investigator: Ellis S. Kempner

Other Investigators: Jay H. Miller

Cooperating Units: None

Man Years (July 1970 through June 1971)

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

An understanding of the control of metabolic activity in unicellular organisms.

Methods Employed:

Culture of microorganisms in the presence of radioactive or other compounds; chemical and physical fractionation of the cellular materials by ultracentrifugation, chromatography and chemical extraction. Measurement of cellular size by conductimetric analysis and video micrography.

Major Findings:

Studies of the protozoan, Euglena gracilis, have been continued.

The final composition of a minimal growth medium has been established. This medium contains the smallest possible levels of all elements which still allow rapid growth of Euglena to high population densities. Measurements of trace contaminants was performed by spark-source mass spectrometry. Commercial glutamic acid was the primary source while both distilled water and the glass containers were shown not to be sources of trace metals. The total quantity of each element present in the medium initially is just about equal to the amount found in the cells at the end of exponential growth phase.

Kinetic studies of the utilization of zinc, manganese, calcium and chlorine were performed by radioactive tracer methods. Except for zinc, all are incorporated proportional to cell growth. Zinc was found to be rapidly depleted from the medium at the initiation of growth, but the cells continued to grow at maximal rate even though no further zinc was available.

Gross chemical analyses (protein, RNA, DNA, lipid, polysaccharide, etc.) and elemental analyses of Euglena were completed. The "free sugars" previously found in Euglena was shown to be the polyalcohol, mannitol. Unfortunately the cells will not accept exogenous mannitol, so that simple tracer experiments are precluded.

The small peptides reported previously have been further characterized. The single most abundant peptide has been isolated. In cooperation with L. Levenbook it was shown to be arginylglutamine, a very basic peptide reported only once before (in the green alga, Cladophora). Other peptides are under examination. All the peptides were found to be metabolically active: they are synthesized from glutamic acid, the sole carbon source, and then coverted to other materials.

Initial studies of Euglena, Hydra and other organisms has begun using time-lapse videomicrography.

Significance to NIAMD Research:

This work is aiding the development of a system for studying differentiated cellular structure in Euglena gracilis with the technical ease usual with bacteria. Using this system, new insight into the structure and function of cells is being gained.

Proposed Course of Project:

1. To study the metabolic relationships of synthetic processes in Euglena to the highly organized cellular structure which has already been elucidated.

2. To extract, separate and identify the peptide materials in Euglena and to establish their role in cellular metabolism.

3. To relate the kinetics of growth with biochemical events.

4. To utilize Euglena to study the role of inorganic ions.

Serial No. NIAMD-LPB-23
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Investigation of the macromolecular organization of living matter.

Previous Serial Number: NIAMD-LPB-24

Principal Investigator: L. W. Labaw

Other Investigators: None

Cooperating Units: None

Man Years (July 1970 through June 1971)

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives:

To gain information about the macromolecules that are essential constituents of living matter and to see how they are arranged in the structures they form. To study certain of these macromolecules, such as viruses and other proteins, in purified form after isolation from the living material. To improve the resolution of the electron microscope and to interpret the way in which images are formed near its limit of resolution.

Methods Employed:

The electron microscopy of microorganisms, cells and tissues in suspension or thinly sectioned. The physicochemical characterization of macromolecular components isolated from such material using electron microscope, x-ray diffraction, and similar established techniques.

Major Findings:

An electron microscopic study of a crystalline human γG1 immunoglobulin has shown the molecules to be somewhere between a Y and a T in outline, when viewed perpendicular to their shortest dimension. This finding agrees with the recent x-ray diffraction results of Dr. David Davies (LMB) and his group, and with previous EM observations on rabbit antibody-hapten

complex by Valentine and Green. A line positioned centrally through the upright part of the Y to T shape and parallel to its length is in the direction of the x-ray determined two-fold rotation axis of the crystal; so each arm of the Y to T shaped molecule must correspond to an Fab portion, and the upright part of tail, to the Fc portion.

This EM study required interpreting micrographs of stained 400 to 900 Å thick crystal sections in which the random "noise" was comparable in size to the periodic "signal." The signal to noise ratios were increased by linearly translating and superposing the micrographs on themselves until the molecular shape could be seen clearly at a magnification of X 10^6.

This study, together with the LMB x-ray diffraction study on similar crystals, gives dramatic confirmation to the popularly accepted shape of the γG immunoglobulin molecule.

Significance to NIAMD Research:

The use of the electron microscope to determine the crystal structure of protein crystals is particularly important when applied to crystals which cannot be grown large enough for a more complete structure study by x-rays.

With increased emphasis on fine structure in electron microscopy, it is pertinent to examine the mechanism of image formation to define the conditions under which one can be assured that observed periodicities on images in the electron microscope have a direct spatial relationship with the object being examined when one is operating near the resolution limit of the instrument.

Proposed Course of Project:

To investigate other protein crystals in the electron microscope using replica, negative staining, and direct transmission techniques to determine their structures.

Publications:

De Nayer, P., and Labaw, L. W.: Identification of large size polyribosomes in bovine thyroid glands. Endocrinology (in press).

Labaw, L. W., and Davies, D. R.: An electron microscopic study of human γG1 immunoglobulin crystals: Preliminary results. J. Biol. Chem. (in press).

REPORTED IN PRESS - 1970

Labaw, L. W., and Olson, R. A.: Further electron microscopic observations of bacteriochlorophyll protein crystals. J. Ultrastruct. Res. 31: 456-464, 1970.

ANNUAL REPORT SUMMARY
LABORATORY OF BIOPHYSICAL CHEMISTRY

The chief interest of this laboratory has been the study of the relationship between structure and function of biological macromolecules. Special emphasis is paid to the proteins involving muscular contraction and blood coagulation, but other systems are also studied when these are better suited to the elucidation of certain aspects of the problem. Advantage is also taken when a pathological condition, either induced in animals or occurring in man, offers new insight into the problem under investigation.

MUSCLE: Use of radioactive ATP has confirmed earlier spectrophotometric studies that ATP binding to myofibrils is Mg-dependent, and suggests two ATP binding sites. (Hotta, Bowen).

Myosin allowed to interact with hydroxylamine forms hydroxamates and, as ultracentrifuge examinations in 4.5 molar urea solution reveal, a cleavage of myosin takes place. From the treated myosin, carboxypeptidase A releases hydroxamated amino acid, which appears to be glutamic acid and presumably originates where the cleavage by hydroxylamine occurred. The observation that myosin may be hydroxamated, with a concomitant cleavage by liver transglutaminase, which is highly specific for glutamic acid, supports this conclusion.

In conjunction with studies in sulfite-oxidase deficiency, tropomyosin B from the muscle tissue of an African civet was prepared. The amino acid composition, optical rotatory dispersion and sedimentation coefficient of this tropomyosin B appear to be very similar to rabbit tropomyosin. (Bodwell, Irreverre and Hornstein).

In glycerinated fibers ATPase activity attains its maximum at the ATP concentration where approximately half-maximum tension develops. (Bowen, Mandelkern). Similarly in actomyosin suspensions, ATPase activity attains a maximum at the ATP concentration where approximately half-clearing occurs. An uncoupling between the energy-supplying and tension-generating processes has been found in myofibril suspensions; it will be important to determine the degree of coupling at progressively more "native" levels of organization-glycerinated fibers and fresh fibers. (Kominz).

The ultimate energy source for muscular contraction is the cleavage of ATP. In order to better understand this molecule, quantum mechanical calculations using the so-called CNDO/2 method were carried out. These calculations explain the fission of the peripheral, rather than the proximal, bond in ATP in terms of charge densities and also demonstrate that a significant part of the energy stored in polyphosphates may be accounted for by the small nuclear rearrangement which occurs when phosphates form polyphosphates. (Alving, Laki).

FIBRINOGEN: The plasma of salmon and steelhead trout yielded fibrinogen fractions capable of clotting upon the addition of bovine thrombin. The amino acid analyses of the peptides released were determined. (Gladner). Lyophilized frog plasma when reconstituted permits the isolation of its fibrinogen. Upon addition of bovine thrombin, a well-formed viscous network of polymer clot was obtained and two arginine-containing polypeptides were found to be released. (Gladner, Osbahr).

It was found that the early products of trypsin action on fibrinogen are quite similar to the plasmin products, and give important clues as to the structure of the fibrinogen molecule. (Marder, Carroll).

HEMOGLOBIN: The model for the action of hemoglobin based on a cooperative effect depending upon shared ionizable groups has been extended to aggregating hemoglobin systems. Data for lamprey hemoglobin are predicted with the same model as that used for mammalian hemoglobin. (Saroff). A study of the Bohr effect in hemoglobin by means of differential titrations in the presence and absence of 2,3-Diphosphoglyceric acid (DPG) was carried out. The changes in the bound hydrogen per mole of hemoglobin upon oxygenation or deoxygenation were measured at different pH values. These experiments show that DPG alters the Bohr effect. (Saroff, Yap).

INFLAMMATION: The isolation of proteases from rat skin induced by injection of anti-inflammatory drugs, such as cortisone, has proceeded. Efforts are directed to the isolation, purification, and specificity of one of these enzymes which rapidly inactivates bradykinin. (Gladner).

BIOPOLYMERS: The physical chemical determination of the molecular weights of several immunopolysaccharides produced by different strains of Diplococcus pneumoniae has been completed. It was found that the behavior is unusual, reflecting a remarkably strong attraction of this biopolymer for the solvent. (Glaudemans, McMaster, Carroll).

A critical analysis of current theories of the hydrodynamic properties of polymers in solution is being carried out in an attempt to relate these properties to the details of local interactions between the monomeric segments, between the segment and adjacent solvent molecules, and between solvent molecules. In this connection, the structure of liquid water and its temperature dependence in relation to its physical properties and the detailed interaction between water molecules is also being studied. (Minton).

Serial No. NIAMD LBC-1
1. Biophysical Chemistry
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemical studies on physiologically important micro and macromolecules in living systems.

Previous Serial No.: NIAMD-LBC 3

Principal Investigator: Filadelfo Irreverre

Technical Assistance: Mr. Edwin Wilson

Cooperating Units: Dr. B. Witkop, LC, NIAMD
Dr. Y. Fujimoto, LC, NIAMD
Dr. J. M. Karle, NRL
Dr. I. J. Karle, NRL

Man Years
Total: 2
Professional: 2

Project Description:

Objectives: Living systems are characterized by their complexity and their myriad of interacting macro and micromolecules. With the use of modern biochemical techniques, some of which were developed in this laboratory, unknown compounds have been observed in physiological fluids and in tissue extracts from various sources including man. Studies have been directed towards the isolation and characterization of some of these metabolites with special emphasis in human subjects such as in cases of mental retardation and metabolic diseases with a view of tracing their biochemical pathways.

Methods: Modern techniques in chemistry and biochemistry have been employed including paper and column chromatography, automated amino acid analysis, thin layer chromatography, gel separation and filtration, paper and gel electrophoresis, UV, infrared and mass spectrometry, NMR, polarimetry, ultracentrifugation, radioisotopic techniques and enzymatic analysis. When necessary, development of new techniques of isolation, detection or micro-assay has been undertaken.

Major Findings: In conjunction with studies in Sulfite-oxidase deficiency in man (covered in previous report), muscle tissue from an African civet, civettictis civetta became available. Tropomyosin B was prepared. From the amino acid composition, optical rotatory dispersion and sedimentation coefficient, the tropomyosin B isolated from the African

civet appears to be very similar to rabbit tropomyosin (Bodwell, Irreverre and Hornstein).

As reported last year, synthesis of cis-3,4-methylene-L-proline, the new natural amino acid from horse chestnuts, and of its trans-isomer, was accomplished.

In order to study the structure and configuration of the 2 diastereoisomeric amino acids, crystals of the hydrochloride of cis-3,4-methylene-L-proline and of the free trans-amino acid were prepared.

Detailed X-ray analyses of the hydrochloride of cis-3,4-methylene-L-proline and of the free trans-amino acid gave complete bond distances, angles and computer stereograms. The bicyclic system approaches a boat conformation both in the cis- and in the trans-acid, a result which confirms the evaluation of the NMR data. The pyrrolidine ring of cis- and trans-acids has all four carbons in a plane, while the nitrogen atom and the cyclopropyl carbon are displaced to the same side of the plane. In this respect, the conformation of the hetero ring differs significantly from all other natural pyrrolidine amino acids. (Fujimoto, Irreverre, Karle and Karle, and Witkop).

The bicyclic ring of the cis- and trans-3,4-methylene-L-proline is rather stable to the action of UV irradiation, heating with various concentrations of HCl including HCl gas under pressure, heating with triglyme at 216°, etc. It was also found that no cyclic imino acids were produced under the above conditions. (Fujimoto and Irreverre).

It was reported (previous report) that hydrolysate of extracts from Koelreuteria paniculata when submitted to a two-dimensional paper chromatography gave a brilliant orange color spot with ninhydrin. This compound has now been isolated. Reaction of the compound with pyridoxal (Kalyankar, G. D. and Snell, E. E., Nature [London] 180, 1069, 1957) and by formation of a copper complex (Crumpler, H. R. and Dent, C. E., Nature [London] 164, 441, 1949) signified that the substance is an α-amino acid. Other evidence suggests the presence of a keto group in the molecule. Chromatographic comparison with 4-oxo-norvaline, a new amino acid synthesized by J. Folk, showed the unknown to have diffetent R_f values. Further work on the structure of this unknown amino acid is being continued. (Irreverre and Wilson).

Significance to Bio-Medical Research and the Program of the Institute: Studies of the structures of newly isolated proteins, new amino acids and metabolites contribute to the ever expanding fabric of knowledge in living systems including man.

Proposed course of project: Biochemical studies of physiologically interesting micro and macromolecules in living systems will continue. Special emphasis will be placed on studies of physiological fluids from patients with metabolic disorders and/or mental retardation with the purpose of isolating and studying unknown compounds and metabolites related to the disease.

Honors and Awards: None.

Publications:

1. Fujimoto, Y., Irreverre, F., Karle, J. M., Karle, I. L. and Witkop, B.: Synthesis and X-ray analysis of cis-3,4-methylene-L-proline, the new natural amino acid from horse chestnuts, and of its trans-isomer. J. Amer. Chem. Soc. 93, 1971.

Serial No. NIAMD LBC-2
1. Biophysical Chemistry
2. Bioenergetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Basic mechanisms of muscular contraction

Previous Serial Number: LBC-4 and 5

Principal Investigator: Dr. D. R. Kominz

Other Investigators: Dr. Wm. J. Bowen (deceased July 26, 1970)
Dr. Remedios M. Avena

Outside Collaboratories: Prof. Leo Mandelkern, Florida State University
Dr. Ken Hotta, Nagoya City Univ., Japan

Man Years
Total: 3.1
Professional: 2.1
Other: 1.0

Project Description:

Objectives: To advance our knowledge of the mechanism of muscular contraction and its regulation at the molecular level.

Methods: In general, studying the enzymatic activity and physical properties of muscle, utilizing model systems of purified contractile proteins, myofibril suspensions, and glycerinated fibers.

Specifically, 1) employing the apparatus of Evans and Bowen with acetokinase as ATP regenerating system, to study simultaneously ATPase and turbidity development in actomyosin and myofibril suspensions, 2) employing the Coulescope particle counter and Intertechnique multichannel analyzer, to study the correlation between length and volume changes in myofibrils, 3) employing chemical P_i determinations to characterize the substrate inhibition of myosin ATPase activity, 4) employing computer facilities to evaluate theoretical models and compare them to experimental data, 5) measuring the tension of glycerinated fibers under varying experimental conditions, 6) utilizing scintillation counter and either centrifugation or filtration to measure binding of radioactive compounds to myofibrils, 7) employing microphotography to determine sarcomere spacing and end-to-end distances of myofibrils, and 8) employing a helium-neon laser to prepare optical transforms of electron micrographs and to measure the effect of ATP on glycerinated fibers.

Major Findings: 1. ATPase activity attains a maximum at the ATP concentration where approximately half-clearing occurs (Kominz). It appears as though ATPase activity may likewise attain its maximum at the ATP concentration

where approximately half-maximum tension of glycerinated fibers occurs (Bowen, Mandelkern). Since it seems unusual that maximum tension should coincide with complete dissociation of actin from myosin, new experimental approaches are required for clarification. Both sets of experiments focus attention on the region of elevated ATP concentration where substrate inhibition occurs.

2. Substrate inhibition in myosin A is not complicated by dissociation of actin. Theoretical analysis of results suggests that Ca-activated myosin A ATPase employs CaATP as substrate, is not activated by Ca, and is inhibited by 1-2 moles of ATP (Avena, Bowen).

3. Methods have been developed for production of myofibrils which do not aggregate at room temperature, and of myofibrils which are linear arrays of rest-length sarcomeres rather than curved arrays of shortened sarcomeres. With myofibril aggregation controlled, electronic sizing procedures indicate an increased protein effective volume of 20-30% upon contraction of myofibrils. Reversal of ATPase activity and turbidity changes of these myofibrils and failure of reversal of contraction and swelling suggests an uncoupling of input and output processes in this system (Kominz).

4. Use of radioactive ATP has confirmed earlier spectrophotometric studies of ATP binding to myofibrils. The ATP binding is Mg-dependent, and suggests two ATP binding sites (Hotta).

Projects Proposed:

It will be attempted to monitor simultaneously on glycerinated fibers the tension, rate of ATP hydrolysis and sarcomere length. In this way the preliminary results concerning ATP-dependence of tension and ATPase activity will either be confirmed or corrected. The effect of the Ca-EGTA regulatory system on these parameters will be explored. Special instrumentation required to monitor the laser pattern is being developed. Special techniques to monitor the ATPase activity of glycerinated fibers will also be required.

During August it is planned that Dr. Ute Groschel-Stewart of Wurzburg, Germany will collaborate in investigating two problems. One is the preparation and sizing of smooth muscle cells; the second is the effect of antibodies on myofibrillar ATPase and turbidity development.

Significance to Bio-Medical Research and the Programs of the Institute:

Studies undertaken on actomyosin or myofibril suspensions suffer from the drawback that they correspond to shortening under zero load; although full contraction of these particles can occur, it is irreversible. Use of muscle fibers allows monitoring under various loads, and allows restretching to the original length. The conventional analogy between turbidity development in model systems and contraction in intact muscle has grave defects; a plausible alternative proposal will be tested on the muscle fibers. An

un-coupling between the energy-supplying and tension-generating processes has been found in myofibril suspensions; it will be important to determine the degree of coupling at progressively more "native" levels of organization --glycerinated fibers and fresh fibers. It is hoped that the two sources of clearing in suspensions--dissociation of actin from myosin and swelling of the myosin--may be distinguished in the more ordered fibers.

Honors and Awards:

None

Publications:

Bowen, W. J. and Mandelkern, L.: The relationship between shortening of glycerinated muscle fibers and ATPase activity. Physiol-Chem. & Physics 2: 47-65, 1971.

Kominz, D. R.: Correlations of length and volume measurements in myofibril suspensions. Biophys. J. 2: 47-65, 1971.

Serial No. NIAMD LBC-3
1. Biophysical Chemistry
2. Macromolecules
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The structure and properties of synthetic polyelectrolytes and some natural polysaccharides as models for charged proteins.

Previous Serial Number: LBC-8

Principal Investigator: Dr. W. R. Carroll

Other Investigators: E. R. Mitchell
H. L. Wolff

Cooperating Units: Dr. V. Marder, Temple University Medical School, Philadelphia, Pa., Dr. P. R. B. McMaster, LMI, NIAMD

Man Years
Total: 3.0
Professional: 3.0
Other: 0.0

Project Description:

Objectives: The main objective of this laboratory is the understanding of the role of charged groups in determining the structure and function of proteins and other naturally occurring macromolecules. Current studies of the behavior of synthetic polyelectrolytes as simplified models are giving us some insight into the influence of charge on polymer structure as well as the possibilities of selective binding and transport of smaller ions in solution and at phase boundaries.

Methods: So far we have limited our methods to those measuring the solution properties of macromolecules. These include: ultracentrifugal analysis, light scattering, electrophoresis, diffusion, nuclear magnetic resonance, activity coefficient measurements using perm-selective membrane cells, optical rotatory dispersion, and other spectral methods.

Major Findings and Proposed Course of Project:

The physical chemical determination of the molecular weights of several immunopolysaccharides produced by different strains of Diplococcus pneumoniae has been completed. In the process of measuring the concentration dependence of diffusion for these substances, it was found that the behavior is unusual, reflecting a remarkably strong attraction of the polymer for the solvent. A theory (Mandelkern-Flory) exists for this behavior, but it has never been tested. These polysaccharides provide a unique opportunity for that test. (Glaudemans, McMaster).

Identification, Hydrodynamic Propertities, and Interaction of the Products of Plasmin and Trypsin Digestion of Fibrinogen: Extending our earlier studies we have now found that the early products of trypsin action on fibrinogen are quite similar to the plasmin products, and give important clues as to the structure of the fibrinogen molecule. (Marder)

Significance to Bio-Medical Research and the Program of the Institute:

Knowledge of the basic chemistry of proteins and their function is a primary requirement for understanding physiological functions and pathological conditions of the body.

Honors and Awards: Dr. W. R. Carroll is a member of the NIH Physiological Chemistry Study Section.

Publications: None

Serial No. NIAMD LBC-4
1. Biophysical Chemistry
2. Physical Biochemistry
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Proteins, enzymes and peptides involved in blood coagulation and inflammation.

Previous Serial No.: NIAMD LBC-4

Principal Investigator: Dr. Jules A. Gladner

Other Investigators: Dr. Albert J. Osbahr
Mrs. P. A. Murtaugh
Mr. Raymond Jacob, Guest Worker

Cooperating Units: Dr. John C. Houck, Children's Hospital, Wash., DC and the George Washington University Medical School, Wash., DC; Dr. John Halver, Chief, Fish Nutrition Laboratory, Fish and Wildlife Service, Dept. of Interior, Cooke, Washington

Man Years
Total: 4
Professional: 4

Project Description:

Objectives: Studies of two systems which are vital to life processes are under investigation. The key step of blood coagulation, the thrombin catalyzed conversion of fibrinogen to fibrin, and all its ramifications constitutes one of studies. In addition, attention has once more been turned to close examination of the physiological properties of peptides released by thrombin upon its action on fibrinogen. Increasing importance is also being given to investigations involving the enzymes, proteins and peptides which appear to play a major role in the biochemistry of inflammation. Both these processes involve the concepts of "limited proteolysis"; i.e., proteolytic enzymes of high specificity, thrombin on the one hand, induced proteases on the other.

Methods: Methods used were those of peptide and protein-enzyme fractionation, purification, and characterization, and protein modification. All methods of physical-biochemistry were employed where needed. Physiological techniques were utilized to examine the biological activity of various peptides. Where applicable, new techniques were developed.

Major Findings: The conversion of fibrinogen to fibrin. Attention

was given to an examination of the action of thrombin upon the fibrinogens of lower species. We compared the fibrinogens, the structure of released peptides and the possible physiological activity of these peptides to the same parameters of certain mammalian species. The plasmas of two fishes, West Coast salmon and West Coast steelhead trout, were utilized. In addition a closer examination of frog plasma was undertaken. It was obvious from initial pilot runs that fractionation of the fibrinogen fractions from fish species did not follow patterns similar to those of the higher species. Clottable fibrinogen fractions could be obtained employing alcohol fractionations, salt and isoelectric precipitations, but yields were low and variable. This appears to be caused by some factors present in the lyophilized plasmas of these fishes. These observations were essentially substantiated by other workers in the field. These fibrinogen experiments are continuing.

When bovine thrombin was added to purified fibrinogen fractions of salmon and steelhead trout, clots of high viscosity were obtained. From the supernate of salmon aggregates (clots), it was possible to obtain two pure peptides. Both were acidic, both Sakaguchi-positive indicating the presence of arginine residues. These were examined as to amino acid analyses and physiological activities. Conventional nomenclature was employed; the fastest moving peptide on high voltage paper electrophoresis was designated A, the slower B.

Amino acid	Peptide A residues	Peptide B residues
Aspartic acid	5-6	1
Threonine	1	-
Serine	-	1
Glutamic acid	2	1
Proline	1	1
Glycine	2	-
Alanine	-	3
Valine	1	1
Leucine	1	1
Tyrosine	1	-
Phenylalanine	-	-
Lysine	2	-
Arginine	1	1
Total residues	17-18	10

Compared to the known structure of peptides from other species, certain structural differences are evident. The A peptide is larger than the B but is only slowly ninhydrin-positive indicating the possibility of a blocked amino terminal which usually occurs for only some of the B peptides. Both peptides contain one arginine residue and these are carboxyl terminal indicating that once again the exact specificity of thrombin for arginine bonds holds.

Both peptides, when examined for physiological activity utilizing the potentiation of bradykinin contraction of smooth muscle, showed no physiological activity at levels assayed. The possibility exists that due to very low yields of these peptides, the concentrations at which they were assayed were too low. These will be carried out at higher concentrations at a future date. (Gladner, Murtaugh).

Lyophilized frog plasma was reconstituted and the fibrinogen fraction removed at 15% alcohol concentration. The fibrinogen was purified and when subjected to analysis in the ultracentrifuge was shown to contain two major components. Preliminary separation of these components on Sephadex G-200 resulted in two fractions which were clottable upon addition of bovine thrombin. A well-formed viscous network of polyner clot was obtained which was similar to previous fibrinogen clots from other species. It is of interest to note that the frog fibrinogen is precipitated from colloidal solution in phosphatebuffer at a 38% ammonium sulfate saturation instead of 25% as observed in other species. This possibly indicates that the frog fibrinogen emulsoid possesses a different charge distribution than the fibrinogen of other species. With the addition of thrombin to the frog fibrinogen, two arginine-containing polypeptides were found to be released concurrent with clot formation. These peptides were separated by high voltage electrophoresis and by ion exchange chromatography on Dowex-50. The amino acid analysis of the peptides was determined. (Osbahr and Gladner).

Biochemistry of inflammation and related reactions. Efforts toward isolation and characterization of a "bradykininase" from rat skin, induced by injection of anti-inflammatory drugs, have continued. This induced enzyme, one of those previously reported, has the property of rapidly inactivating bradykinin, a nonapeptide present in many cases of stress such as inflammation, infection, burn, etc. Since it has not been possible to induce this enzyme in other animal skins, a thorough investigation of conditions for obtaining maximum levels of enzyme activity in rat skin was undertaken as to dosage, time before sacrifice, etc. Since there are many experiments we wish to carry out concerning the exact specificity of this unusual enzyme, it was decided to make the isolation and purification of substantial quantities of this enzyme the primary goal. We hope to do this prior to utilization of many of the scarce synthetic peptide substrates we have in our possession. All these peptides contain the sequence phenylalanine-serine, which is the bond hydrolyzed by the enzyme in its action on bradykinin. (Gladner, Houck).

Significance to biomedical research and the program of the Institute: Very little is known concerning the biochemistry of anti-inflammatory compounds and inflammation. It is most interesting to speculate that one of the actions of anti-inflammatory compounds is to induce an enzyme(s) which can destroy the activity of bradykinin, a smooth muscle contractor and pain producer which appears whenever conditions of stress, such as infection, inflammation, burns, even pregnancy, etc., are prevalent. In addition to the physiological implication, in utilizing these unique enzymes, it will be

possible to study certain enzyme mechanisms, especially as applies to limited proteases. This type of enzyme is becoming more prevalent as physiological systems of protein-protein interactions are studied more closely.

Proposed course of project: It is proposed that all three proteases induced by injection of anti-inflammatory drugs in rat skin will be isolated and characterized. The major effort is to be placed on the isolation of the enzyme which is a "bradykininase" but resembles chymotrypsin.

Further studies will be carried out on fish fibrinogens.

Honors and Awards: None.

Publications: Houck, J. C. and Gladner, J. A.: Proposed mechanisms for the ameliorative effects of corticosteroids in shock. Shock: Biochemical, Pharmacological and Chemical Aspects. New York, Plenum Press, 1970, pp. 125-132.

Serial No. NIAMD LBC-5
1. Biophysical Chemistry
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Electrons in Biomolecules.

Previous Serial No.: NIAMD LBC-10

Principal Investigators: R. E. Alving
K. Laki

Cooperating Units: J. Ladik, City University of New York

Man Years
Total: 1.25
Professional: 1.25

Project Description:

Objectives: The improved quantum mechanical methods which have been developed in the past decade (such as extended Huckle and CNDO) have been applied only occasionally to molecules of interest to biochemists. Even when biomolecules are investigated, the questions answered are usually more of interest to physicists than to biochemists. Our objective in applying quantum mechanical methods to ATP is to investigate in a semiquantitative way the interaction that has been proposed between the phosphate ends of the molecule with a view to explaining the instability of the molecule in water. We also hope to show ways in which ATP can interact with proteins in a way which could transmit energy from ATP breakdown into the protein. This problem can be approached by calculations done on ATP and part of a protein molecule considered as a unit.

Methods: The 360/65 computer at NIH was used when possible, and for computations requiring large amounts of core, the NASA 360/95 was necessary. The CNDO program used is a modification of one written at Carnegie Institute of Technology.

Major Findings: We have found that in the unionized molecule, the electronic states of the pusine, ribose and phosphate groups are independent of one another, while the ionized form distributes some charge to the peripheral atoms as would be postulated from Pauling's electroneutrality principle.

The calculated electronic distribution in ribose diphosphate offers an explanation of the hydrolysis site in ATP. Perturbation of bond lengths

shows that less energy is required to stretch the peripheral bridge bond, and this is the bond known from O^{18} studies to be broken during hydrolysis. This is explainable in terms of charge distribution.

The calculated charge on the phosphorus atoms in polyphosphates is highly dependent on the net molecular charge and this offers a possible connection between electron shifts in cells and ATP hydrolysis. Electron shifts in a macromolecule complexed to ATP could cause expansion or contraction of local orbitals abd thereby trigger splitting of bonds.

A new factor contributing to the energy stored in pyrophosphates has been discovered in the small rearrangement of molecular structure which occurs when orthophosphate is incorporated into pyrophosphate. This rearrangement of the fully ionized species is calculated to require 20 K cal. The magnitude of this energy change would be attenuated somewhat by water, but it is certainly a significant factor in explaining the large free energy change in ATP hydrolysis.

Significance of biomedical research and the program of the Institute: Once electron distribution in a molecule is known, other properties of the molecules may be calculated. Only in this way can intramolecular details of chemical reactions and biophysical phenomena such as muscle contraction be studied.

Proposed course of project: The results obtained will be used to explain biophysical phenomena.

Honors and Awards: None.

Publications: None.

Serial No. NIAMD LBC-6
1. Biophysical Chemistry
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Cleavage of myosin and fibrinogen by hydroxylamine

Previous Serial No.: None

Principal Investigator: K. Laki

Technical Assistance: Edwin Wilson

Man Years:
Total: 1.0
Professional: 1.0

Project Description:

Objectives: It has been demonstrated that hydroxylamine cleaves collagen or ribonuclease preferentially at an asparagine residue followed by a glycine residue. This project is an exploration of whether hydroxylamine brings about a similar cleavage of myosin and fibrinogen.

Methods: Methods applied in this project involved the use of tritium labelling, scintillation counters, paper and column chromatography, UV spectrometry, ultracentrifuge analysis and others.

Major Findings: It is now well established that in the case of ribonuclease and large fragments of collagen, hydroxylamine causes a cleavage between asparagine and glycine residues with a concomitant uptake of hydroxylamine into the carboxyl group of asparagine. According to Bernstein, at this location in the peptide chain, a rearrangement takes place through the formation of a 5-membered ring involving the β-carboxyl of asparagine.

As a result, in addition to the original asparagine residue, aspartic acid may arise (to a certain extent) with the peptide chain shifting to the β-position. Similar considerations may be applied to glutamine residue in the chain. In our experiments we examined (1) the extent to which myosin and fibrinogen take up hydroxylamine; (2) whether these proteins become tritiated with tritiated water at an asparagine (or glutamine) residue other than C-terminal, to indicate a shift of the peptide bond to the β- (or γ-) position; (3) to see if a cleavage can be demonstrated.

We found that both myosin and fibrinogen incorporated hydroxylamine. We also found that carboxypeptidase removed most of the hydroxylamine-bound amino acids (presumably aspartic or glutamic acid).

The fact that incubation with tritiated water in the case of myosin tritiated not only the C-terminal residues (isoleucine, histidine), but also aspartic and glutamic acids indicates that a shift of the peptide chain to β- or γ-position has occurred. Ultracentrifuge experiments in 4.5 molar urea indicate that a cleavage of the molecule has taken place.

Preliminary experiments indicate that in the case of myosin, the cleavage occurred at a glutamic acid residue. When at low concentrations of hydroxylamine, transglutaminase is added to the incubation mixture, myosin becomes hydroxamated and ultracentrifuge experiments indicate a cleavage. Since transglutaminase is specific for glutamic acid residues, these observations also support the tentative conclusion that cleavage takes place at a glutamic acid residue in myosin.

Significance to Bio-medical Research and the Program of the Institute: Since muscle tissue contains transglutaminase, a locus where the peptide chain may be shifted to the γ-position may have an important bearing on how the sliding of the thin filament attached to the head of myosin occurs.

Proposed Course of Project: Effort is being made to identify unequivocally the amino acids which become hydroxamated.

Honors and Awards: None.

Publications:

1. Laki, K.: Actin. In Laki, K. (Ed.): Contractile Proteins and Muscle. New York, London, Marcel Dekker, 1971, pp. 97-107.

2. Laki, K.: Flagellin. In Laki, K. (Ed.): Contractile Proteins and Muscle. New York, London, Marcel Dekker, 1971, pp. 135-154.

3. Laki, K.: Size and shape of the myosin molecule. In Laki, K. (Ed.): Contractile Proteins and Muscle. New York, London, Marcel Dekker, 1971, pp. 179-217.

4. Laki, K.: Tropomyosin A. In Laki, K. (Ed.): Contractile Proteins and Muscle. New York, London, Marcel Dekker, 1971, pp. 273-288.

5. Laki, K. and Benkő, S. A.: The effect of transamidase on myosin. Kísérletes Orvostudomány (Experimental Medical Science) 22: 48-57, 1970.

Serial No. NIAMD LBC-7
1. Biophysical Chemistry
2. Macromolecules
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The elucidation of the structure and interactions of biologically important macromolecules.

Previous Serial Number: LBC-7

Principal Investigator: H. A. Saroff

Other Investigators: Dr. R. B. Simpson
Dr. William Yap

Cooperating Units: Dr. Arthur Spector, University of Iowa Clinical Research Center, Iowa City, Iowa; Dr. J. Preer, Federal City College, Washington, D.C.

Man Years:
Total: 3-1/2
Professional: 3
Other: 1/2

Project Description

Objectives: The main objective of this laboratory is an elucidation of the structure of naturally occurring macromolecules based on the methods of physical chemistry. Macromolecules are studied primarily in solution and the emphasis is on the accumulation of data on the state of the molecules with respect to environment in solution. Gross features of structure, as well as fine detail, may be observed in the study of macromolecules in solution.

Methods: At present, we are concerned with methods of observing macromolecules in solution. In our studies aimed at finer details of protein structure, the methods used involve the measurements of the binding of small molecules or ions to specific sites on the protein molecule, nuclear magnetic resonance (on model compounds, so far), optical rotatory dispersion measurements, and other spectral techniques. For other properties of the protein molecules, we employ the ultracentrifugal, light scattering, electrophoretic and diffusion techniques.

Major Findings and Proposed Course of Studies: We studied the Bohr effect in hemoglobin by means of differential titrations in the presence and absence of 2,3-Diphosphoglyceric acid (DPG). The changes in the bound hydrogen per mole of hemoglobin upon oxygenation or deoxygenation were

measured at different pH values. For dialyzed hemoglobin sample, the H+ released on oxygenation reached a maximum value of about 1.9 mole H+/mole Hb at pH 7.5. The dialyzed sample contains 1.4 mole DPG/mole Hb. When the hemoglobin sample was deionized by passing through an ion exchange column, the DPG content became less than 0.1 mole/mole Hb, and the maximum of the differential titration curve shifted to about 7.25, with a maximum H+ released of about 1.5 mole/mole Hb. When 1 mole DPG/mole Hb was added to the deionized hemoglobin sample, the maximum of the differential titration curve shifted to about pH 7.6 and the maximum is 1.9 H+ released per Hb. Thus, DPG alters the Bohr effect. To make a more complete description, we are studying also the binding of hydrogen and metal ions to DPG. We found that DPG binds 2 sodium with the binding constant of the first site having a value of about $10^{2}.8$. DPG binds sodium ions with constants greater than those for pyrophosphate. (Yap).

The model for the action of hemoglobin based on a cooperative effect depending upon shared ionizable groups has been extended to aggregating hemoglobin systems. An example of such a system is Lamprey hemoglobin. Data for Lamprey hemoglobin are predicted with the same model as that used for mammalian hemoglobin with changes only in the constants used for aggregation of the subunits and ionization of the Bohr sites. (Saroff).

The role of the SH groups of hemoglobin has been studied very extensively in a number of laboratories. Our model for the action of hemoglobin does not include the SH groups in an explicit manner but some experimental studies are underway in an attempt to provide a basis for the role of the SH group in the action of hemoglobin. (Simpson, Saroff).

Studies are being continued on the use of computational models for the binding of ions to clusters of ionizable groups in model compounds. (Preer, Saroff).

One of the important functions of serum albumin is the transport of fatty acids. In order to elucidate the binding of fatty acids to albumin, we (Arthur Spector, Richard B. Simpson) continued our investigation of the association constant of the fatty acid with its anion. Two structures for the dimers of fatty acids in aqueous solution have been proposed, one in which the carboxyl groups are hydrogen bonded, and the other in which the hydrocarbon chains form hydrophobic bonds. The dimerization constants are being measured by means of conductivity and partition to determine which structure predominates in each fatty acid. (Simpson).

Significance to Biomedical Research and the Programs of the Institute: Our emphasis is on the relationship between chemical properties and the structure of macromolecules. Once these properties are properly related, the attack on the relationship between structure and function can proceed on a solid basis. The proteins and macromolecules studied are either enzymes or those of interest in biological systems. Our systems are highly controlled with reference to the conditions of the protein environment and the biomedical significance of our findings derive from extropolation to the

biological system.

Honors and Awards: None.

Publications:

1. Dygert, S. L., Muzii, Giovanna and Saroff, H.A.: The ionization of clusters. I. The dicarboxylic acids. J. Phys. Chem. 74: 2016-2026, 1970.

2. Saroff, H.A.: Model for the action of hemoglobin. Proc. Nat. Acad. Sci. USA 67: No. 4, 1662-1668, 1970.

3. Yap, W. T. and Saroff, H. A.: Application of the ising model to hemoglobin. J. Theor. Biol. 30: 35-39, 1971.

Serial No. NIAMD LBC-8
1. Biophysical Chemistry
2. Macromolecules
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Intermolecular Interactions in Physical Biochemistry

Previous Serial No.: None

Principal Investigator: Allen P. Minton

Man Years:
Total: 1/2
Professional: 1/2

Project Description:

Objectives: The general subject of the investigation is dealt with in three types of systems: a) the nature of the interactions between water molecules and their influence upon local structure and physical properties in liquid water, b) solute solvent interactions in solutions of polymers and biological macromolecules and their influence upon solution properties, and c) interactions between subunits and their relation to cooperative behavior in oligomeric proteins.

Methods: The main approach in this area is theoretical. Data to be rationalized are derived from such measurements as viscosity, diffusion and light scattering.

Major Findings and Proposed Course of Studies:

a) Water: Quantitative relations developed in this laboratory are used to study the effect of detailed local structure in a homogeneous solid or liquid upon the dielectric and optical behavior. This relation is used with structural data for ice to predict dielectric and optical properties and conversely with dielectric and optical properties for water to obtain information about the local structure in the liquid. The dielectric results support a picture of liquid water as consisting of large solid-like domains, the boundaries of which migrate rapidly throughout the volume accounting for the small structural relaxation times and liquidity of the substance. The same results are inconsistent with the idea of water as clusters of small numbers of H-bonded molecules.

b) Polymer Solutions: The major theories of thermodynamic and hydrodynamic behavior of polymer solutions are being critically reviewed in an attempt to remedy known shortcomings and to quantify the concept of solvent "goodness" in terms of the energetics of solute-solvent interaction.

c) Subunit interaction in proteins: A recently published attempt to distinguish between two types of models for cooperative behavior (sequential and two-state allosteric) in the case of the oxygenation behavior of hemoglobin has been shown to proceed from fallacious assumptions. The treatment used was shown to be incapable of distinguishing between the two models in this case.

Significance to Biomedical Research and the Programs of the Institute: Since the behavior of biological molecules is profoundly influenced by interactions with water, a knowledge of structural properties and interactions in water and aqueous solutions of biological macromolecules is clearly a prerequisite for understanding life processes on a molecular level. Knowledge of the way in which subunit interactions relate to cooperative behavior in oligomeric proteins appears to be fundamental to an understanding of metabolic control mechanisms.

Honors and Awards: None.

Publications: None.

ANNUAL REPORT SUMMARY

Laboratory of Molecular Biology

The work of the Laboratory of Molecular Biology continues to be concerned with the explanation of biological processes at the molecular level. The problems under investigation in the laboratory include x-ray diffraction and physical chemical studies of proteins and nucleic acids, biochemical studies of enzymes important to nucleic acid metabolism, genetic and biochemical studies of the mechanisms and regulation of protein synthesis in bacteria and mammalian cells, and of transport in bacteria, and genetic studies of the physiology of bacteria phage. In several of these areas major breakthroughs have been made by members of the laboratory during the past year.

DNA-tryptone Interaction and Chromatin Structure.

A careful analysis has been made of the binding of histones to DNA. It was observed that at moderately high salt concentration the binding was freely reversible and that even those protein fractions that remain continue to be bound and are capable of free exchange and rearrangement. Since nearly all previous studies of the properties of partly dissociated chromatin have been carried out in such solvents, these observations show that no conclusions can be drawn from such studies about the role of various histone fractions in chromatin structure and function.

This investigation shows that more than half of the chromatin DNA is exposed and chemically reactive which is contrary to the commonly held view that most of the DNA of chromatin must be covered up by proteins. The result suggests that the action of chromatin must occur by blocking only limited regions of the DNA, i.e. the promotor "regions" to which the RNA polymerase must bind in order to initiate transcription. The methods developed in this investigation should make it possible to isolate such regions. (Felsenfeld, Clark).

Genetics and Structure of the Oncogenic Virus SV-40.

A temperature sensitive SV-40 mutant has been isolated and characterized. An analysis of the location of the block in the vegetative cycle caused by this mutation suggests that the block may involve the uncoating of the virus.

A preliminary investigation indicates that after infection by the virus a high molecular weight protein component appears prior to the appearance of any viral components. The characterization of this protein and of the SV-40 DNA core protein complex has been undertaken. (Martin, R. G., Robb, J.A., Medrano, L., McCann, P.).

Biochemical Control Mechanisms in Histidine Biosynthesis.

A frameshift mutation in the second gene, hisD of the histidine operon of S. typhimurium has been characterized. The sequence of that portion of the mRNA corresponding to the end of this gene has been determined. The results demonstrate that intercistronic regions definitely exist and suggest that such regions play a role in the initiation of translation. Revertants of this mutant have been shown to result in a chimera enzyme possessing the activities of both the hisD gene and the adjacent hisC gene. (Martin, R. G., Rechler, M., Bruni, C. B.)

Molecular Structure.

1. Immunoglobulin structure

The three dimensional structure of the cryoglobulin DOB has been determined at 6 Å resolution. The molecule exists in the form of a T with the two Fab regions connected to the Fc region by a thin ribbon of electron density. These results are in striking confirmation of the predictions of others from physical chemical and electron microscopic studies of immunoglobulin structure.

2. The structure of gamma chymotrypsin and its inhibitors

Refinement of the three-dimensional of gamma chymotrypsin has been continued. The investigation of a number of oligo peptide chloroketone inhibitors of the type acetylalanylphenylalanyl chloroketone have been carried out. These inhibitors attach to the histidine 57 of the molecule and it has been demonstrated that the mode of binding involved a series of subsites, S_1, S_2 and S_3 which bind the peptides P_1, P_2 and P_3 proceeding from the C terminus of the inhibitor. These subsites are formed principally by the presence of an extended piece of polypeptide chain in the proteins from Ser 214 to Gly 216 which binds the inhibitor with the same type of hydrogen bonding that one sees in the antiparallel pleated sheet conformation. It is believed that this represents the mode of binding of the acetyl enzyme intermediate in the chymotrypsin catalyzed cleavage of polypeptide chains.

3. The investigation of the crystal structure of an acid protease from Rhizopus chiniensis has been commenced.

The molecule belongs to the general class of proteases which are active around pH 4, e.g. pepsin, rennin. A determination of the crystal structure of this enzyme should throw considerable light on the mechanism of action of this important class of enzymes. (Dr.R. Davies, Sarma, R., Cohen, G., Silverton, E., Segal, D., Braxton, H., Swan, I.)

The Mechanism of Genetic Recombination.

Mutants of E. coli defective in the DNA ligase have been examined. They show high UV sensitivity and fail to act as hosts for recombination-deficient mutants of lambda. These results are being studied for the information they yield with respect to the mechanism of recombination. Ligase mutations are being mapped on the E. coli chromosome. (Gellert, M., Hicks, M.L).

A study of recombination in physically defective bacterial phage lambda has shown that lambda DNA requires both cohesive ends in order to participate in productive site-specific recombination. (Cohen, G.)

This result is supported by the observation that defective lambda particles whose DNA has one cohesive end cannot undergo site-productive sites-specific recombination. (J. W. Little, in collaboration with M. Gottesman).

Protein Synthesis in Mammals.

The molecular weight of native transferase II has been determined to be about 90,000. Aminoacyltransferase I, the soluble factor responsible for the binding of aminoacyl tRNA to ribosomes during polypeptide chain elongation has been extensively purified. (Maxwell, Collins, Tudor, Moon).

Studies on Polynucleotide Structure.

A number of physical chemical investigations on the structure of polynucleotides have been carried out by members of the laboratory.

1) The investigation of the conformation of single-stranded polynucleotides in solution have been continued. A single-strand polydeoxynucleotide has been prepared (apurinic acid) in which about half the purines are missing from their usual points of attachment to the sugar ring. It is found that removal of nearly half of the bases has no effect upon the rigidity polynucleotide, and it is concluded that the bases do not play an important role in confering rigidity. In addition the substitution of deoxyribose for ribose does not cause collapse of the polynucleotide chain, indicating that the presence of the 2'-hydroxyl groups in polyribonucleotides do not cause additional rigidity. (Felsenfeld, G., Achter, G., Stannard, B. J.).

2) The heats of reaction of various substituted poly rC polymers complexed with poly rI have been measured. A methyl group substituted at the 5-position of the cytidine ring contributes a stabilizing effect to the enthalpy of at least 700 cal/mole of base pairs. A bromine atom substituted in this position produces a stabilization of at least 2 kcal/mole of base pairs. When the magnitude of this enthalpic stabilization is compared with the overall stabilization indicated by the increase in melting temperature, it follows that an unfavorable compensating entropy change must be associated with the reaction of the two polyribonucleotide chains combining to form the two-stranded complex. (Ross, P.D., Scruggs, R. L., Howard, F. B., Miles, H.T.).

3) The formation of polynucleotide aggregates at acid pH have been studied. Although the aggregates generally appear amorphous, they sometimes exhibit a highly irregular appearance. They are being examined for evidence of internal structure. (Zimmerman, S.B., Coleman, N.S.)

4) It has been shown that tetraalkylammonium salts effect a drastic modification of the phase diagrams for complexes formed between poly rA and poly rU [and also with the related polymers rT and r (U,T)]. The dependence of T_m in the 2 ——> 1 transition of the U,T copolymers, has been shown to be linear with the Rt content of U,T copolymer. Similar effects are observed with methylcytidine and bromouracil. Laser Raman spectra of many polynucleotide helices have been studied and related to the infra red absorption bands. The results are being also correlated with theory. (Miles, H.T., Howard, F. B., Ishakawa, F., Ikeda, K., Lewis, T., Frazier, J.).

Serial No. NIAMD-LMB-1
1. Laboratory of Molecular Biology
2. Section on Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Enzymatic Joining of DNA Strands and its Role in Genetic Recombination.

Previous Serial Number: NIAMD-LMB-6

Principal Investigator: Martin Gellert

Other Investigators: Gerald Cohen
John W. Little
Minnie L. Hicks

Cooperating Units: M. Gottesman, Section on Microbial Genetics, Laboratory of Molecular Biology

Man Years:

Total:	4
Professional:	3
Other	1

Project Description:

Objectives: To understand the physiological role of DNA ligases; to study the biochemical basis of genetic recombination.

Methods Employed: Ultracentrifugation, bacterial transformation, standard enzymological and microbiological techniques.

Major Findings: 1) Mutants of E. coli defective in DNA ligase (M. Gellert, M. L. Hicks): We have extended our work on these mutants along several lines.

a) Our original isolates were double mutants, which were overproducers of a damaged DNA ligase (see last year's project report). We have now succeeded in separating these two mutations, thus obtaining a strain whose ligase activity is further depressed. This strain has roughly normal ligase activity at 20°, but only 1% of this level at 42°. The strain is UV-sensitive but otherwise grows well at 42°, with a doubling time not appreciably different from that of the parental strain. The existence of this mutant raises, in heightened form, the question of whether DNA ligase is an essential enzyme. If it is not, the current model of DNA replication in E. coli may need drastic revision.

b) This mutant has some interesting interactions with phage λ. Recombination-deficient (red) mutants of λ do not grow on it at 42°, and other

λ phages make clear plaques on this strain at 42°, indicating a much reduced frequency of lysogenization. These observations are being followed up.

c) The location of ligase mutations on the E. coli chromosome is being mapped. Preliminary results show that both the defective and overproducer mutations map between thy and his, in a region which contains other DNA- related functions. More detailed mapping by P1 transduction is now in progress. We want to ge able to transfer the mutations into other strains, and also to use P1 transduction as a means of generating more and better ligase mutants.

2). Recombination in Physically defective bacteriophage λ DNA (G. Cohen).

A study of recombination in physically defective λ DNA has been completed. A Kaiser-Hogness helper assay was employed to examine recombination in both whole and defective λ DNA; this assay allows an alternative approach to the standard procedure of studying recombination in λ, in which cellular infection occurs through the intact phage rather than its DNA. The virtue of this approach is that it permits study of the effects not only of gene mutation on recombination but also the effects of considerable physical modification to the λ genome. Three classes of physically defective DNA molecules were obtained in the controlled mechanical breakage of λ DNA by hydrodynamic shear left and right half molecules having the AB^+ and imm^{λ} genetic markers, respectively, and molecules approximately three quarters the length of λ DNA which retain the imm^{λ}-AB^+ lined markers. Each of these three classes of DNA molecules bears a single cohesive end and are, therefore, unable to circularize.

Recombination between helper and infecting DNA in the assay was measured in conditions where λ specified recombination functions were provided solely by the infecting DNA. Whole DNA molecules participate productively in both Int (site-specific) and Red (generalized) promoted recombination. Left half molecules, which bear neither of the recombination genes, have no recombination activity. Right half molecules engage in productive Red promoted recombination but were totally unable to participate in productive site-specific recombination. The third class of defective λ DNA molecules showed a behaviour similar to that of the right half molecules. The defect in the right half molecules could not be attributed to physical damage to the Int system or to an inadequate level of its activity. The defect was remedied by providing generalized recombination functions, so that a novel coupling of too recombination recombination systems led to productive site-specific recombination in right half molecules. The results show that λ DNA requires both cohesive ends in order to participate in productive site-specific recombination. This is interpreted as meaning a requirement for circularity.

3). Studies of defective λ particles whose DNA has one cohesive end (J. W. Little, in collaboration with M. Gottesman).

We completed the studies, described in detail in last year's report, concerning the abnormal particles produced upon induction of a λ prophage which cannot be excised normally. Briefly, we showed previously that two classes of particles are produced: one class, termed λdocR, carries phage and bacterial genes to the left of the prophage center on DNA molecules with only a right cohesive end, and can express its genes when assayed as intact phage. The other class, termed λdocL, carries phage and bacterial genes to the right of the prophage center on DNA molecules with only a left cohesive end, and is incapable of expressing its genes as intact phage--it is detected only by assay of extracted DNA.

The functional difference between the two types has been further explored. We found that when the defective particles were made in the absence of phage tails, the λdocR heads, like normal λ heads, were unstable, while the λdocL's were stable. Moreover, the range of sizes of DNA molecules which can be packaged in a λdocL head is much greater than that of λdocR heads. These facts, taken together with the inability of λdocL's to express their genes, lead us to hypothesize that perhaps the right cohesive end plays an important role in normal phage injection; instability of normal phage heads would be a by-product of this role which ordinarily would not be deleterious to the phage since tail-less mutants are a laboratory freak.

We also found genetic evidence that DNA circularity plays an important role in the site-specific recombination in λ, since λdocR DNA cannot undergo such recombination productively. However, when a second, general recombination event elsewhere can take place, site-specific recombinants are found. They have undergone a second event, whose topological role, we believe, is to generate a circle.

A preparative method for making highly-labeled ^{3}H-DPN (J. W. Little).

In the course of studies with DNA ligase, we devised a simple scheme for preparing nicotinamide-labeled ^{3}H-DPN by the following exergonic reactions:

1. glucose-1-^{3}H + ATP $\xrightarrow{\text{hexokinase}}$ glucose-6-P-1-^{3}H + ADP

2. G-6-P-1-^{3}H + TPN^{+} $\xrightarrow{\text{G-6-P dehydrogenase}}$ TPNH-^{3}H + H^{+} + 6-phosphogluconate

3. TPNH-^{3}H $\xrightarrow{\text{alkaline phosphatase}}$ DPNH-^{3}H

4. DPNH-^{3}H + pyruvate + H^{+} $\xrightarrow{\text{lactic dehydrogenase}}$ DPN^{+}-^{3}H + lactate

Since dehydrogenases add and remove protons specifically from one side of the nicotinamide ring or the other, the dehydrogenases in steps 2 and 4 are chosen to have opposite stereospecificity, so that the labeled hydrogen atom is retained in step 4.

Our previous preparation was rather rough-and ready; we have worked out optimal conditions for a high yield. The virtues of this scheme are that it is simple--the intermediates are not isolated--and yields a product of very high specific radioactivity (3 c/mmole) in high yield.

4). Role of gene 32 product in phage T4 metabolism (J. W. Little)

Gene 32 of phage T4 codes for an essential protein, termed 32 protein, which binds specifically and co-operatively to denatured DNA. It is implicated in vivo in DNA replication and recombination. It is required in large amounts; indirect evidence suggests that the phage burst size is proportional to the amount of 32 protein. Hence, if a means were available to limit severely the amount of 32 protein, and thus the burst size, one might select for mutants which were better able to grow under such restrictive conditions.

We have employed the following system to effect this type of restriction. Normally, chain termination in E. coli is coded by the codon UAA. A strain which can read a UAA codon as an amino acid is termed an ochre suppressor; it must suppress infrequently, or it would be unable to terminate its polypeptide chains efficiently. By contrast, suppressors of the amber nonsense codon, UAG, may be much more efficient, because UAG is not the normal chain terminator. Typical efficiencies of suppression are 3-5% and up to 70%, respectively. Ochre suppressors can suppress both ochre and amber mutations, at this low frequency (efficient amber suppressors do not suppress ochre mutants).

As amber mutants of gene 32 are available, it is possible to limit severely the amount of 32 protein by growing them in ochre-suppressing strains. They do not make plaques. However, we have found that, at low frequency, second-site mutants arise which make small plaques on these strains. We are beginning to characterize their nature. We hope that such a study will aid in understanding DNA replication and recombination in T4, in particular by revealing interactions among various proteins and processes. While speculation is premature, several non-trivial possibilities for the nature of these mutants are one or more of the following:

1. They might require less 32 protein than normal T4.

2. They might express gene 32 more efficiently than normal T4.

3. They might be able to use, at least in part, the (hypothetical) bacterial protein which carries out the same function for E. coli. This possibility is made more attractive because T4 32 protein interacts specifically with T4 DNA polymerase, so possibly the mutants might make a polymerase which interacts with the host "32 protein".

Publications: Gellert, M., and Bullock, M. L. DNA ligase mutants of E. coli Proc. Nat. Acad. Sci. 67, 1580-1587 (1970).

Serial No. NIAMD-LMB-2
1. Laboratory of Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Protein Synthesis in Mammals: Mechanisms and Metabolic Controls

Previous Serial Number: NIAMD-LMB-3

Principal Investigator: Elizabeth S. Maxwell

Other Investigators: James F. Collins
Elizabeth Tudor
Hong Mo Moon

Man Years
Total: 3 1/2
Professional: 3 1/2

Project Description:

Objectives: Our overall objective is to gain an understanding of the mechanisms and metabolic controls of protein synthesis in mammals. We hope to obtain a partial understanding of these processes through detailed knowledge of the soluble enzymes involved in polypeptide chain initiation, elongation and termination.

Methods Employed: All of the methods employed are either standard procedure or minor modifications thereof.

Major Findings: This year work has continued on aminoacyltransferases I and II , the two soluble enzymes necessary for polypeptide chain elongation in rat liver, and some preliminary studies have been carried out on chain initiation in this system.

Last year we reported on the enzymatic properties of transferase II which was purified to homogeniety. Diphtheria toxin catalyzes the transfer of the adenosine diphosphate ribose moiety (ADPR) of NAD^+ to a covalent linkage on transferase II. Unlike the native enzyme, the ADPR derivative of transferase II is inactive in overall polypeptide chain elongation, in a model system measuring translocation of peptidyl tRNA and in the ribosome-dependent hydrosis of GTP.

The molecular weight of native transferase II has been determined by a variety of methods to be about 90,000. The ADPR derivative contains about one mole of ADPR per mole of enzyme. Both the native enzyme and its inactive derivative consist of single polypeptide chains. Upon aging

at 0 - 5°, both forms undergo a specific breakdown to three components of molecular weights around 64,000, 42,000 and 37,000. Attempts to duplicate degradation by chemical and enzymatic treatments have been unsuccessful. Experiments with ^{3}H-ADPR derivatives of the enzyme indicate that ADPR is present in the undegraded enzyme and in one or both of the smaller components, but not in the component of molecular weight 64,000. Both the native enzyme and a fragment(s) can accept ^{3}H-ADPR, but no evidence for a fragment active in amino acid incorporation or in ribosome-dependent hydrolysis of GTP was obtained.

Aminoacyltransferase I, the soluble factor responsible for the binding of aminoacyl tRNA to ribosomes during polypeptide chain elongation has been extensively purified. The best preparations, greater than 50% pure, act catalytically in amino acid incorporation but apparently only stochiometrically in binding of phenylalanyl tRNA and methionyl $tRNA_M$ to ribosomes in the presence of poly U and poly AUG respectively. This elongation-factor cannot bind the mammalian initiating species of methionyl $tRNA_F$ to ribosomes. Preliminary evidence indicates that the 1 M KCl ribosomal wash contains a factor which can bind this initiating species to ribosomes.

Significance to NIAMD research: The enzymatic mechanisms in protein synthesis in mammals and the metabolic control of these processes is of major interest to biology and biochemistry. Our emphasis so far is on enzymatic mechanisms. Hopefully, understanding of control will follow.

Proposed course of project: We propose to continue investigation of the enzymatic steps involved in peptide chain initiation, elongation and extension in mammalian systems. The possibility of metabolic control at each step investigated will be considered.

Specifically, we propose to continue investigations of the physical and chemical characteristics of homogeneous transferase II with the hope of furthering the understanding of its mechanism of action. Studies are underway to determine its amino acid composition and its aminoterminal and carboxyterminal residues. Isolation and characterization of a tryptic peptide containing ^{3}H-ADR is expected to lead to identification of the site of linkage of ADPR and perhaps to some insight into possible mechanisms of metabolic control of the enzyme.

Further attemps to obtain homogeneous transferase I will be made. If a homogeneous preparation can be obtained, more studies of its enzymatic and physicochemical properties will be made.

Preliminary experiments on polypeptide chain initiation in the system from rat liver will be expanded. The use of mammalian viral RNA as messenger is anticipated in these studies.

Publications:

Moon, H. M., Collins, J. F., and Maxwell, E. S.: Discrimination by rat liver aminoacyltransferase I against Met-tRNA$_F$. Biochem. Biophys. Res. Comm. 41, 170-176 (1970).

Raeburn, S., Collins, J. F., Moon, H. M., and Maxwell. E. S.: Aminoacyltransferase II from rat liver. I Purification and enzymatic properties. J. Biol. Chem. 246, 1041-1048 (1971).

Collins, J. F., Raeburn, S. and Maxwell, E. S.: Aminoacyltransferase II from rat liver. II Some physical and chemical properties of the purified enzyme and its adenosine diphosphate ribose derivative. J. Biol. Chem. 246, 1049-1054 (1971).

Serial No. NIAMD-LMB-3
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Genetics and structure of the oncogenic virus SV40

Previous Serial Number: NIAMD-LMB-8

Principal Investigator: Robert G. Martin (6/12)

Other Investigators: Dr. James Robb (12/12), Dr. Leandro Medrano (12/12)
Dr. Peter McCann (7/12)

Man Years:

Total:	4-1/12
Professional:	3-1/12
Other:	1

Project Description:

Objectives: To analyze the genetic structure of the oncogenic virus SV40 through the isolation of conditional lethal mutants. Interaction between host functions and viral replication will also be investigated. Attempts will be made to obtain mutated host cells with suppressor mutations which allow growth of the temperature sensitive viral mutants at the non-permissive temperature. Structural analyses of the DNA-protein complex of the viral core will be undertaken.

Major Findings: A temperature sensitive SV40 (Robb & Martin) mutant has been isolated and characterized. The mutant appears to be normal in its ability to adsorb to host cells. The virus itself is not temperature sensitive. The block appears to be early in the vegetative cycle since all known early functions are prohibited at the non-permissive temperature, i. e. there is no appearance of "V" antigen, "T" antigen, and no induction of host or viral DNA synthesis. It is postulated that the block may involve the "uncoating" of the virus. Consistent with this hypothesis are the findings that when DNA from the temperature sensitive mutant is employed for infection, single cycle growth is not inhibited at the non-permissive temperature and other SV40 mutants which are mutually complementary fail to complement the new mutant.

The binding of SV40 protein to DNA cellulose columns (Medrano & Martin) has been examined. Preliminary results indicate that after infection a high molecular weight protein component appears prior to the appearance of any viral components. The characterization of this component is under investigation.

Characterization of the SV40 DNA core protein complex (McCann & Martin) has been undertaken. The DNA-protein complex is obtained by standard procedures. Physical chemical characterization has been undertaken. It appears that greater than 95% of the DNA in the complex is sensitive to degradation by staphylococcal nuclease. The remaining DNA is protected from degradation by the core protein. The digested complex has a sedimentation coefficient of approximately 4S. The entire complex can be made insensitive to degradation by reaction with poly D lysine. A detailed characterization of the core is proceeding.

Significance to Bio-medical Research and the Program of the Institute:

The significance of this project is that it may lead to some insights into carcinogenesis and other metabolic diseases.

Publications:

Robb, James A.: Microcloning and replica plating of mammalian cells. Science 170: 857-858, 1970

Serial No. NIAMD-LMB-4
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemical control mechanisms in histidine biosynthesis

Previous Serial Number: NIAMD-LMB-7

Principal Investigator: R. G. Martin (6/12)

Other Investigators: Dr. Matthew Rechler (12/12), Dr. C. Bruno Bruni (12/12).

Man Years

Total:	3 1/2
Professional:	2 1/2
Other:	1

Project Description:

Objectives: Little information exists concerning intercistronic regions (the spaces between genes) in bacteria. Characterization of an unusual mutant in the his operon of Salmonella typhimurium should indicate definitively whether such regions exist and possibly lead to some understanding of the functions of such regions.

Major Findings: The Intercistronic Region - (M. Rechler, C. Bruno Bruni, R. Martin). A frameshift mutation in the second gene, hisD of the histidine operon of S. typhimurium has been characterized. This mutant is unusual in that the enzyme corresponding to hisD remains active. As a result of sequencing the -COOH terminal portions of the wild type and mutant enzymes, and knowning the genetic code, it has been possible to determine the sequence of that portion of the mRNA corresponding to the end of the hisD gene. Further characterization of this mutant seems to indicate that intercistronic regions definitely exist and suggests that such regions play a role in the initiation of translation.

Revertants of this mutant have been analyzed (Rechler and Bruni) and found to have fused the hisD gene to the adjacent hisC gene. Enzymological studies on this fused enzyme indicate that the K_ms for both reactions are unaltered although the v_{max} for the dehydrogenase (hisD) is reduced.

Analysis of the amino terminal sequence of the wild type transaminase and mutant transaminase are underway.

Significance to NIAMD Research: Our understanding of gene function is fundamental to an understanding of genetic disease and prerequisite to genetic manipulation.

Publications:

Rothman-Denes, L., and Martin, R. G.: Two mutations in the first gene of the histidine operon of Salmonella typhimurium affecting control. J. of Bact. 106: 227-237, 1971.

Rechler, M. M. and Bruni, C.B.: Properties of a fused protein formed by genetic manipulation. Histidinol dehydrogenase-imidazolylacetol phosphate-L-glutamate aminotransferase. J. Biol. Chem. 246: 1806-1813, 1971.

Serial No. NIAMD-LMB-5
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Interactions among replicons in E. coli: The bacterial chromosome, a fertility factor, and temperate bacteriophage

Previous Serial Number: NIAMD-LMB-9

Principal Investigator: Michael B. Yarmolinsky (12/12)

Other Investigators: Dr. Judah L. Rosner (12/12), Dr. David I. Friedman (5/12)
Dr. Toshiya Takano (6/12).

Collaborators at the Institut de Biologie Moleculaire, Paris 5:

Aline Brachet, Danièle Schwartz

Man Years

Total:	2-2/3
Professional:	2-2/3
Other:	0

Project Description:

Objectives: An understanding, eventually in molecular terms of regulatory mechanisms which control the replication of various bacterial replicons, their specific rearrangements, and their distribution between daughter cells. Specifically our attention has been focused on:

a) Characterization of bacterial mutants with altered capacity for propagation of lambdoid phages.

b) Genetics and physiology of bacteriophage P1.

c) Investigation of a bacterial mutant affecting bacteriophage plasmid formation.

d) Viral suppression of mutations causing conditional defects in bacterial chromosome replication.

Major Findings:

a) Characterization of bacterial mutants with altered capacity for propagation of lambdoid phages.

In the previous annual report we communicated the isolation by Dr. David Friedman of bacterial mutants that survive induction of λP^- prophage. Since $\lambda N^- P^-$ prophage do not kill on induction, it seemed possible that viral N function specifically fails to be effective in these mutants. Dr. Friedman has accumulated genetic evidence that this is the case. The N protein of λ is believed to interact with RNA polymerase or with a chain-terminating factor in transcription, called ρ. Dr. Friedman sought to determine whether the bacterial mutations he has selected are located at the site of known RNA polymerase (rifampicin-resistance) mutations. Since leaving NIH Dr. Friedman has confirmed his preliminary indications that his mutations do not map next to rifampicin-resistance markers. This finding is of great interest because it suggests the existence of a new genetic site controlling RNA polymerase, ρ, or other transcription factors. Friedman's mutants appear to be in a class distinct from phenotypically similar mutants described by Georgeopoulos.

b) Genetics and physiology of bacteriophage P1.

Bacteriophage P1 lysogenizes E. coli without integrating into the chromosome. The linear DNA injected into the cell by the mature phage is converted into a circular form which then replicates as a plasmid. Dr. Rosner's studies on P1 lysogeny, making use of a thermoinducible mutant of P1 which also carries a determinant for chloramphenicol resistance($P1CM^R ts$), have yielded the following information: (1) Lysogenization is equally efficient following single or multiple infection with P1. (2) The efficiency of lysogeny is 10 times greater in rec^+ than in rec^- cells. (3) Spontaneous prophage curing occurs in both rec^+ and rec^- lysogens at a rate of about 10^{-5}/cell/generation. (4) Upon superinfection of rec^+ or rec^- P1 lysogens by appropriately marked P1, substitution of the infecting phage for the resident prophage is observed. (5) Superinfection of rec^+, but not rec^- lysogens, yields a substantial portion of doubly lysogenic bacteria. These strains are relatively unstable and revert to single lysogens.

Thus, circularization of P1 appears to occur in rec^- and rec^+ cells even if they are lysogens. Accordingly, this is accomplished by a host function other than rec or by a non-repressible P1 function.

The observations are consistent with, and support, the hypothesis (suggested for the sex factor, F, by Jacob, Brenner and Cuzin) that the P1 plasmid requires a unique cellular (membrane?) site for prophage replication. This site may be competed for by superinfecting P1 giving rise to prophage substitution. Two prophages could not replicate in the same cell unless both were attached to the same site; a feat which may be accomplished by a rec-promoted recombination between the two P1 circles resulting (reversibly) in a single, double-sized circle. Physical evidence for such a molecule is being sought.

Dr. Rosner has also been studying various defective P1 lysogens. (1) Starting with $P1CM^R ts$ lysogens, chloramphenicol-resistant, thermostable variants have been selected (about 3×10^{-6}/cell). These cells carry various P1 genes, including P1 immunity, but do not produce viable phage. It is

possible that these cells carry defective P1 which have integrated in the chromosome. This hypotheses is supported by the finding (Yarmolinsky and Schwartz, below) that similarly obtained variants suppress bacterial DNA defects. (2) By selecting for resistance to very high concentrations of chloramphenicol, a strain has been isolated which appears to carry one copy of the CM^R gene on the bacterial chromosome and another copy on the P1 prophage. When cured of the prophage, the strain is resistant to low levels of chloramphenicol but when relysogenized with $P1CM^R$, they become again resistant to high levels. (3) A $P1CM^R$ts lysogen which produces no viable P1 ($< 10^{-10}$ pfu as compared with a normal lysogen) has been fortuitously isolated by Dr. M. E. Gottesman. When cured, this strain adsorbs P1 and may be killed by the P1 or lysogenized by it but no viable phage are produced. The newly relysogenized strain is killed by thermal induction but neither lyses nor, if lysed artifically, produces any viable phage. We are particularly intrigued with the possibility that in this strain P1 DNA replication may occur in the prophage state but not in the vegetative state. For all of these defective strains, attempts will be made to map the defects and to determine their nature.

c) Investigation of a bacterial mutant affecting bacteriophage plasmid formation.

In previous work, Dr. Takano demonstrated that lon mutants of E. coli do not allow plasmid formation by bacteriphage P1 or by an N gene mutant of bacteriophage λ, yet these phage are capable of normal vegetative growth on lon bacteria. Since arriving at the NIH, Dr. Takano has isolated a mutant of P1 that can persist as a plasmid in the lon bacteria. However, when lon bacteria are simultaneously infected with normal P1 and the P1 mutant, only the mutant P1 persists. Thus the mutant is unable to supply the normal P1 with the function needed for plasmid formation. The nature of this cis-dominant effect is under investigation.

d) Viral suppression of mutations causing conditional defects in bacterial chromosome replication.

Interest in functions that intervene in the replication of DNA and in the coordination of cell division with this replication has been stimulated recently by the finding that certain normally gratuitous replicons can complement (suppress) certain conditional defects in bacterial DNA synthesis. The suppression of bacterial thermosensitive mutations in the initiation of DNA synthesis (e.g. mutation T46) appears to require physical integration of the defective replicon into the non-defective replicon. Among the replicons capable of effecting "integrative suppression" are the sex factor F and temperate bacteriophages λ, P2, and P1. Because P1 is the only known bacteriophage maintained normally as a plasmid, we have taken a particular interest in the conditions which permit this phage to effect integrative suppression. Danièle Schwartz and Michael Yarmolinsky have shown that the bacterial recA function is required for integrative suppression by P1. This result, although consistent with results of analogous experiments on integrative suppression

by F, is somewhat surprising in view of the presumed existence of a P1 function (like erf of P22) that catalyzes intraphage recombination in a rec⁻ host.

When a thermoinducible P1CM^R (constructed by J. L. Rosner) is used to select for P1 mutants that fail to kill their host upon induction, it is found that a considerable percentage (up to 50%) of the resulting defective lysogens have become capable of colony-formation at high temperature despite the presence (prior to lysogenization) of thermosensitive defect in initiation of DNA replication. Three surprising conclusions may be drawn (1) The efficiency with which P1 DNA integrates into the chromosome is not negligible, although P1 does not have any efficiently used chromosomal location. (2) Integration of P1 frequently results in loss of lethal genetic structures or loss of the capacity to express genes which can confer lethality. (3) The additional requirements for integrative suppression by P1 must be minimal because the suppression is exceedingly efficient for non-lethal induced P1. The general validity of this last conclusion has received striking support from experiments in which integrative suppression is accomplished with λN⁻. David Friedman has shown that induction of λN⁻ is not lethal provided the bacterium is simultaneously infected with the same phage. Philippe Brachet has recently shown that the conditions for preventing λN⁻ lethality described by Friedman are sufficient to allow up to 1% of the infected T46 lysogens to survive as colony-formers at a temperature where uninfected non-lysogens fail to replicate their DNA.

A second class of bacterial DNA replication mutants, whose members arrest DNA synthesis immediately upon passage to the non-permissive temperature, have previously resisted attempts to suppress them in trans or in cis. Aline Brachet has shown that P1 inefficiently suppresses a mutant in this second class (T266). When P1 is isolated from such suppressed bacteria and used to make lysogens of T266, suppression is 100%. Moreover, P1 transferred to T266 from an Hfr carrying defective P1 which are non-lethal when induced, also effects 100% suppression. The suppression can be reversed by displacement of the mutant P1 with wild type P1. The mutant P1 is effective in trans, being unaffected by a recA⁻ mutation introduced prior to infection.

One explanation for these observations currently being tested is that P1 can reverse the T266 defect by preventing the leakage of a diffusible factor required to maintain DNA replication. This hypothesis is supported by an observation of Kohiyama that thymidine uptake into DNA in toluene-treated cells of T266 can be restored by addition of a low molecular weight substance. Furthermore, changes in cell surface characteristics due to P1 are suggested by observations of Yarmolinsky on the sensitivity to glucose of certain defective P1 lysogens.

Significance to Biomedical Research and the Program of the Institute: Perhaps the most important and unexplored processes in molecular biology are DNA synthesis and cell division. Insofar as this research contributes to our understanding of these processes as they occur in bacteria, it will have significance for Bio-medical Research and the Program of the Institute.

Specifically, it should be mentioned that bacteria and their bacteriophages and sex-factors are in many cases the vectors of disease and of resistance to disease. Furthermore, DNA synthesis and cell division in bacteria are more amenable to study than the corresponding processes in mammalian cells, for which they may provide helpful models.

Proposed Course of Project: Each research project, with the exception of Friedman's project (a), will be continued in this laboratory. Particular emphasis will be placed on obtaining genetic information concerning the signals which coordinate the replication and segregation into daughter cells of host and plasmid replicons.

Publications:

Kass, Leon R. and Yarmolinsky, Michael B.: Segregation of functional sex factor into minicells. Proc. Nat. Acad. Sci. 66: 815-822, 1970.

Friedman, David I.: A bacterial mutant affecting λ development. In The Bacteriophage Lambda. (ed) A. D. Hershey, et al (in press).

Yarmolinsky, Michael B.: Making and joining DNA ends. In The Bacteriophage Lambda. (ed) A. D. Hershey, et al (in press).

Serial No. NIAMD-LMB-6
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Bacterial control mechanisms and carbohydrate transport

Previous Serial Number: NIAMD-LMB-11

Principal Investigator: Mark Levinthal (12/12)

Other Investigators: Jen Yeh (9/12)

Man Years

Total:	1-3/4
Professional:	1-3/4
Other:	0

Project Description:

Objectives: Pi strains - We are asking three questions: 1) How does the Pi event lead to expression of his genes? 2) What is the nature of the Pi replicative machinery which allows the Pi episome to replicate autonomously? 3) What is the genetic consequences of cis diploidy for part of the histidine region?

Transport Studies: We have concentrated on the genetics, physiology and control of melibiose metabolism. Melibiose is one of the sugars not utilized by phosphotransferase mutants.

Major Findings: Pi strains: 1) Pi must be integrated in order to be transferred by an Hfr strain. Using an Hfr containing a hisD temperature sensitive lesion, I have shown the frequency of integration is 1 in 10^3 chromosomes transferred.

2) Pi integration involves the entire Pi factor. Using an Hfr containing hisD23, an amber mutation, I have been able to recover the hisD23 mutation from integrated recipients.

3) Pi arises from hisG203 by a non-Campbell excision event. However, segregation of Pi from the integrated state is a Campbell excision event.

Melibiose Metabolism: I have developed assay procedures for α-galactosidase and mel permease. I have characterized both activities and studied induction and catabolite repression.

Mapping is almost complete. Mel$^-$ mutants are very close to metA.

Significance to bio-medical research and the program of the institute:

None

Proposed course of research:

Pi strain: 1) Analysis of the segregants from the integrated Pi strains.

2) Further analysis of Pi integration and excision in recA strains.

Melibiose metabolism: 1) Isolate mel$^-$ control mutants.

2) Do cis/trans tests with these mutants.

3) Purify α-galactosidase from constitutive mutants.

4) Study inducibility of mel enzymes in car strains.

Publications:

Levinthal, Mark. Biochemical studies of melibiose metabolism in wild type and mel mutant strains of Salmonella typhimurium. J. Bact. 105: 1047-1052, 1971.

Serial No. NIAMD-LMB-7
1. Laboratory of Molecular Biology
2. Section on Molecular Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: X-ray Investigation of the Structure of an Intact Immunoglobulin

Previous Serial Number:: NIAMD-LMB-13

Principal Investigator: D. R. Davies
V. Sarma
E. W. Silverton
D. Segal

Cooperating Units: Dr. William Terry, National Cancer Institute, Immunology Branch

Man Years:

Total:	2-1/2
Professional:	2-1/2
Other:	0

Project Description:

Objectives: The structure of a human γG1 immunoglobulin is being examined at 6 Å resolution by the isomorphous heavy atom replacement method. Attempts are also being made to obtain crystals of other immunoglobulins suitable for X-ray examinations.

Methods Employed: X-ray diffraction analysis with the heavy atom isomorphous replacement method.

Major Findings: During this last year an electron density map has been calculated for the immunoglobulin (Dobbs) at a resolution of 6 Å. Examination of the electron density map showed that within the region occupied by one molecule the electron density appeared to be divided into three globular regions. Two of these are identical and are related by the operation of the crystallographic twofold rotation axis. The other region is unique and lies on the twofold rotation axis, we have therefore identified it as the Fc part of the molecule, the other two regions corresponding to the Fab parts.

Since at this degree of resolution neighboring molecules will produce a bridge in the electron density map that may be mistaken for the bridge joining the Fc to the Fab regions, it has been difficult for us to make an unequivocal assignment of the overall shape of the molecule. However we have examined the various ways in which the Fc and Fab moieties might be

joined together and of these we favor one structure in which the molecule has the overall shape of a T.

A number of other heavy atom derivatives have now been discovered and data have been collected for some of these. We intend shortly to compute another electron density map which should have a significantly improved signal to noise ratio. This will permit us to examine the outline of the molecule in more detail.

We have attempted to crystallize the homogeneous rabbit antibodies of the kind obtained by Krause (from material given to us by Dr. R. Krause). One of these preparations showed small crystals but we have not yet obtained crystals suitable for X-ray diffraction investigation. We have also crystallized the Fc portion of the molecule from rabbit.

Significance to Bio-medical Research and the Program of the Institute: Elucidation of the structure of an immunoglobulin will be a major step in revealing the mechanism of interaction between antibody and antigen.

Proposed Course of Project: We are continuing our research for additional heavy atom derivatives for this protein and also we are continuing attempts to crystallize antibodies and fragments of antibodies.

Publications:

Sarma, V. R., Silverton, E. W., Davies, D. R., and Terry, W. D.: The three dimensional structure at 6 Å resolution of a human γG1 immunoglobulin molecule. J. Biol. Chem., in press.

Davies, D. R., Sarma, V. R., Labaw, L. W., Silverton, E., Segal, D., and Terry, W. D.: X-ray diffraction and electron microscope studies on a crystalline human immunoglobulin. In Annals of New York Acad. Sci., Symposium on Immunoglobulins, 1971, in press.

Serial No. NIAMD-LMB-8
1. Laboratory of Molecular Biology
2. Section on Molecular Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: X-ray Diffraction Investigation of γ-chymotrypsin and Other Hydrolytic Enzymes.

Previous Serial Number: NIAMD-LMB-12

Principal Investigator: David R. Davies
Gerson Cohen
David Segal
Ian Swan

Other Investigator: Hazel Braxton

Cooperating Units: None

Man Years

Total:	4-1/2
Professional:	3-1/2
Other:	1

Project Description:

Objectives: The determination of the 3-dimensional structure of γ-chymotrypsin and an investigation into its mechanism of action. The crystallization and examination of a number of other hydrolytic enzymes.

Methods Employed: X-ray diffraction analysis with the heavy atom isomorphous replacement method.

Major Findings: 1. X-ray diffraction investigation of γ-chymotrypsin. During this year we have been concerned with the refinement of the crystal structure of tosyl-γ-chymotrypsin at 2.7 Å resolution. The model has been carefully rebuilt to fit the electron density map using the optical comparator. The coordinates are in the process of refinement.

2. Investigation of inhibitors bound to γ-chymotrypsin. The results reported in preliminary fashion in the last report have been extended. Crystals of γ-chymotrypsin inhibited with a) acetyl-alanyl-glycyl-phenylalanyl-methyl chloroketone (AAGPCK), b) acetyl-alanyl-phenylalanyl-methyl chloroketone (AAPCK), and c) acetyl-phenylalanyl-methyl chloroketone (APCK), have been examined by the difference Fourier technique. Although these inhibited crystals are not isomorphous with the native protein crystals, nevertheless by examination of difference maps between various inhibitors it has been

possible to locate the position of the inhibitor on the enzyme. The finding reported last year that the inhibitor forms a complex antiparallel sheet arrangement of hydrogen bonds with the polypeptide backbone of the enzyme has been confirmed. It has been demonstrated (by Kraut et al.) that the active site of subtilisin, a bacterial serine protease has a virtually identical arrangement of interaction with the inhibitor, thus extending the generality of this result.

Measurements of the rate of hydrolysis of oligopeptides of specific sequence have been compared with the predictions based on this model, and have been shown to be completely consistent with it.

3. The acid protease from Rhizopus chinensis has been crystallized in a form suitable for X-ray diffraction. Previous work by others has demonstrated that the enzyme is closely related to the acid proteases pepsin, and renin. We have commenced a crystal structure analysis of this enzyme with the confident expectation that it will shed considerable light on the mechanism of this important class of acid proteases.

Significance to Bio-medical Research and the Program of the Institute: A knowledge of the 3-dimensional strucdure of proteins is an essential prerequisite for an understanding of the molecular mechanism of action of enzymes.

Proposed Course of Project: While the structure determination of γ-chymotrypsin and its inhibitors is being brought to a close, more weight will be given to the crystal structure analysis of the acid protease from Rhizopus chinensis.

Publications:

Davies, D. R., and Segal, D. M.: Protein Crystallization: micro techniques involving vapor diffusion. In Jakoby, W. (Ed.): Methods in Enzymology. New York, N. Y., Academic Press, 1971, in press.

Davies, D. R., and Doctor, B. P.: tRNA Crystallization. In Cantoni, G. L., and Davies, D. R. (Eds.): Procedures in Nucleic Acid Research. Vol. II. New York, N. Y., Harper & Row, 1971, in press.

Serial No. NIAMD-LMB-9
1. Laboratory of Molecular Biology
2. Section on Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Structure of Single Strand Polynucleotides in Solution

Previous Serial Number: NIAMD-LMB-15

Principal Investigator: Gary Felsenfeld

Other Investigators: E. Achter
B. J. Stannard

Cooperating Units: None

Man Years

Total:	2.5
Professional:	1.5
Other:	1

Project Description:

Objectives: To elucidate the structure of single strand polyribonucleotides in solution.

Methods Employed: Theoretical analysis, light scattering, viscometry, spectrophotometry, sedimentation velocity, sedimentation equilibrium.

Major Findings: We have continued our investigation of the conformation of single strand polynucleotides in solution. In earlier studies we showed that even where the bases of a single strand polyribonucleotide are not stacked upon one another, the molecule is a highly extended structure. There is some reason to believe that this rigidity arises from restrictions in rotation about the bonds of the polynucleotide backbone, but conclusive experimental proof of this is still lacking.

In order to show that the observed rigidity is conferred by the backbone and not by the bases, we have prepared a single strand polydeoxynucleotide, apurinic acid, in which about half the bases (the purines) are missing from their usual points of attachment to the sugar ring. Direct measurement of the optical and hydrodynamic properties of our samples shows that there is no stacking interaction between the remaining bases, and that there is no hydrogen bonding between bases. After preparing well-defined molecular weight fractions, we studied the dependence of sedimentation velocity of apurinic acid upon molecular weight under a variety of temperature and solvent conditions. In this way we were able to find ideal solvent conditions,

in which the unperturbed dimensions of the molecule can be measured. We find that the unperturbed apurinic acid molecule sediments at a rate that is nearly identical to that of polyribouridylic acid of the same degree of polymerization (after correcting for differences in the average nucleotide mass). Thus, removal of nearly half of the bases, with consequent destruction of most possibilities for base-base interaction, has no effect upon the rigidity of the polynucleotide. We conclude that the bases do not play an important role in conferring rigidity. A further conclusion is that the substitution of deoxyribose for ribose does not cause collapse of the polynucleotide chain, i.e., that the presence of 2'-OH groups in polyribonucleotides does not cause additional rigidity.

We have also begun work on the properties of single strand stacked polynucleotides at low temperature. Although it is known that polyriboadenylic acid (poly rA) forms stacked, ordered structures at low temperature, the order is not sufficiently 'long range' to permit X-ray diffraction studies. We have prepared low molecular weight poly rA fractions (100 to 400 nucleotides per molecule) and measured their radius of gyration by light scattering at temperatures down to -14°C. At these temperatures most of the poly rA molecules have very few unstacked bases, and it is possible to extrapolate the data to obtain dimensions of the stacked form. Our preliminary results are consistent with the presence of a single strand helical structure having a repeat of 3.6 to 3.8 Å per nucleotide.

Significance to Bio-medical Research and the Program of the Institute: The structure of polynucleotides in solution is directly related to function. In transfer RNA, for example, the folding of the molecule appears to be related to its biological function, while the ability of DNA to act as a template for DNA replication or messenger RNA synthesis depends upon the ability of DNA to undergo structural changes. Our finding that polyribonucleotides are (quite apart from the rigidity provided by base stacking) extraordinarily rigid molecules is quite important in understanding the rules that govern polynucleotide structure formation. Thus, theories concerning the processes of strand separation and combination in multistrand structures such as DNA must now take this property into account, since it has a profound influence upon these processes.

Proposed Course of Project: Detailed studies of polynucleotide dimensions in nonaqueous solvents will begin shortly. These should help determine both the role of short-range electrostatic forces, and of solvent structure, in polynucleotide structural stabilization. Work is also being carried out on synthetic polydeoxynucleotides to determine what differences, if any, exist between ribose and deoxyribose containing polymers.

Publications:

Achter, E. K., and Felsenfeld, G.: The Conformation of Single-Strand Polynucleotides in Solution: Sedimentation Studies of Apurinic Acid. Biopolymers. In press.

Serial No. NIAMD-LMB-10
1. Laboratory of Molecular Biology
2. Section on Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: DNA-Histone Interaction and Chromatin Structure

Previous Serial Number: NIAMD-LMB-14

Principal Investigator: Gary Felsenfeld

Other Investigators: Robert Clark

Cooperating Units: None

Man Years:

Total: 1 1/2
Professional: 1 1/2

Project Description:

Objectives: The objective of this project is to study the interactions of histones with DNA, in order to determine the structure of chromatin and the role of protein-DNA interaction in the regulation of DNA activity in the nucleus.

Methods Employed: Spectrophotometry, analytical ultracentrifugation, dialysis equilibrium, and other standard techniques are used to study the binding of charged molecules to DNA, and the composition of the regions of DNA to which these molecules are bound. Standard chromatographic and electrophoretic techniques are used in purification and identification of histones.

Major Findings: We have studied the structure of chromatin by measuring its susceptibility to digestion by DNase I and staphylococcal nuclease. At low salt concentrations, where chromatin proteins are tightly bound to DNA, we find for chromatin from a variety of sources that slightly more than half the DNA is digestible. Identical results are obtained with native chromatin, and with chromatin in which protein and DNA are crosslinked with formaldehyde before digestion. In the former case, no measurable protein is released into solution.

In order to have an independent measurement of the open regions of chromatin, we have also carried out titrations of chromatin with polylysine at low ionic strength. We find once again that slightly more than half the DNA is available for combination with polylysine without displacement of any

Publications:

Clark, R. J., and Felsenfeld, G.: Structure of Chromatin. Nature New Biology 229: 101-106, 1971.

chromatin protein. Furthermore, the regions that are digestible are the same as those that can be titrated: as polylysine is added to chromatin, decreasing amounts of DNA become available for digestion in a completely reciprocal way.

Since the ultimate goal of our study is to isolate regions of chromatin DNA covered by various chromatin protein fractions, we are also interested in whether the chromatin proteins are bound irreversibly to the DNA, or whether they are capable of undergoing exchange and rearrangement. This question can be answered by mixing chromatin and radioactively labelled DNA (isolated from the same organism), digesting with nuclease, and determining whether any of the labelled DNA is protected from nuclease attack. We find that under the conditions we use for our digestion and titration studies the histones are bound irreversibly. If the chromatin is exposed to NaCl concentrations of 0.6 M or higher, however, even those protein fractions that remain tightly bound are capable of free exchange and rearrangement. Studies of the properties of partly dissociated chromatin are commonly carried out in such solvents; our observations show that no conclusions can be drawn from such studies about the role of various histone fractions in chromatin structure or function.

Our investigation shows that more than half of chromatin DNA is exposed and chemically reactive. This is contrary to the commonly held view that most of the DNA of chromatin must be covered by protein, since chromatin is such a poor template for action of DNA-dependent RNA polymerase. If the restricted template activity is in fact the result of the presence of chromatin proteins, the proteins must act by blocking only limited regions of the DNA - e.g. the "promoter" regions to which the RNA polymerase must bind to initiate transcription. The digestion and exchange methods we have developed should make it possible to isolate such regions, if they exist.

Significance to Bio-medical Research and the Program of the Institute: Chromatin is the DNA-protein complex isolated from the nuclei of eukaryotic organisms. On the basis of chromatin's reduced priming activity in vitro as a template for DNA-dependent RNA polymerase, it has been suggested by many investigators that the proteins of chromatin are responsible for the selective reduction in genetic activity that gives rise to cellular differentiation. The mechanism of cellular differentiation is one of the major problems of modern biology. The methods we have developed make it possible to isolate the various protein fractions attached to their original binding sites in chromatin. It is our hope that this will permit elucidation of the role of chromatin proteins in differentiation.

Proposed Course of Project: Individual DNA segments covered by various histone fractions will be isolated and studied. The relationship between degree of 'exposure' of DNA and availability as a template for DNA-dependent RNA polymerase will also be studied, using chromatin prepared under conditions that do not give rise to rearrangement of bound proteins. The purpose of these studies is to determine which protein fractions, if any, are responsible for restriction of the template activity of chromatin DNA.

Serial No. NIAMD-LMB-11
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Thermodynamics of Interactions involving Polyribocytidylic Acid and some of its derivatives with Polyriboinosinic Acid.

Previous Serial Number: NIAMD-LMB-17

Principal Investigator: Philip D. Ross

Other Investigators: Robert L. Scruggs

Cooperating Units: Dr. F. B. Howard, Dr. H. T. Miles

Man Years

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives: To delineate, in thermodynamic terms, the effect of variation of chemical structure upon the stability of base pairing in ordered nucleic acid structures.

Methods Employed: The heat of reaction of each type of complex is observed calorimetrically and is studied as a function of salt concentration and temperature. In this investigation, the thermodynamic parameters associated with the reaction forming the 1:1 helical complex between the common substrate polyriboinosinic acid (poly rI) and polyribocytidylic acid (poly rC) and various derivatives of poly rC, containing substituents in the 5-position of the cytidine ring are being studied. Copolymers of poly rC and their derivatives containing substituents at the 5-position of the pyrimidine ring are also being examined to determine the effect of copolymer composition upon the heats, entropies and free energies.

Major Findings: A methyl group substituted at the 5-position of the cytidine ring contributes a stabilizing effect on the enthalpy of at least 700 cal/mole of base pairs in the poly rMeC·poly rI complex. The bromine group substituent at the 5-position of the cytosine ring produces a very large enthalpic stabilization of at least 2 kcal/mole of base pairs in poly rBrC·poly rI. The result obtained with the copolymers, a linear dependence of ΔH upon copolymer composition, rules out the occurrence of any Br - Br; neighbor interactions.

The magnitude of this enthalpic stabilization in both cases exceeds the overall stabilization of five per cent, (a 20° increase in the melting temperature of these complexes over that of unsubstituted poly rC), hence it follows that an unfavorable compensating entropy change is associated with the reaction of the two polyribonucleotide chains combining to form the two-stranded complex.

The different values of ΔH found in this work for these different 5-substituted polyribocytidylic acids show that the heats and entropies of the interaction between different nucleic acid base pairs may be different. These examples are especially convincing since the differences between the various polynucleotide species are at the minimal level of a single substitution.

Significance to Bio-medical Research and the Program of the Institute: These measurements provide fundamental thermodynamic information on the energetics of base pairing interactions in nucleic acids. Studies of the interactions between poly rI and poly rC and its 5-substituted derivaties are timely, in light of the medical interest in the properties of poly (rC + rI) as an interferon inducing substance.

Proposed Course of Project: This work is completed.

Serial No. NIAMD-LMB-12
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Aggregation of Polynucleotides at Acid pH.

Previous Serial Number: None

Principal Investigator: Steven B. Zimmerman

Other Investigator: Norma F. Coleman

Man Years

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives: To describe and hopefully better understand the mechanisms involved in the aggregation of polynucleotides at acid pH.

Methods Employed: Standard techniques of biochemistry and biophysics.

Major Findings: The basic observation is that a wide variety of polynucleotides, including homo-and heteropolymeric deoxy-and ribonucleic acids, become aggregated below about pH 5; the aggregates are easily sedimented by low centrifugal forces. The aggregates are generally amorphous when examined in the light microscope; however under certain, as yet, poorly defined conditions, aggregates from several polynucleotides have highly regular appearances, including needles or very thin irregular plates. The aggregates go into solution above ca. pH 7; enzymatic degradations of such redissolved aggregates indicate no covalent changes are introduced by the aggregation procedures. These initial experiments hence suggest a relatively general reversible pH-dependent phase transition. Whether novel secondary structures are prerequisite or concomitant to aggregation remains to be seen.

Significance to Bio-medical Research and the Program of the Institute: Polynucleotide interactions are of prime importance in cellular regulation and in many of the most basic synthetic processes in normal and abnormal metabolic states. The current system offers potential insight into certain aspects of polynucleotides interactions.

Proposed Course of Project: Descriptive studies to gather data on the physical and chemical properties of the aggregates and the conditions leading to their formation and destruction. Attempts to produce suitable samples of the more ordered aggregates to examine by X-ray and birefringence techniques.

Serial No. NIAMD-LMB-13
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Reversible Thermal Inactivation of Bovine Pancreatic Deoxyribonuclease.

Previous Serial Number: NIAMD-LMB-16

Principal Investigator: Steven B. Zimmerman

Other Investigator: Norma F. Coleman

Man Years

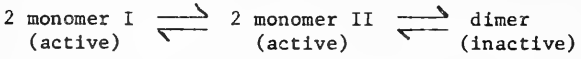
Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives: To better understand the mechanisms of action and of inactivation and stabilization of pancreatic DNase.

Methods Employed: Standard techniques of enzymology and physical chemistry.

Major Findings: The reversible thermal inactivation of bovine pancreatic DNase at acid pH can be rationalized in terms of the following conversions:



The properties of these species and of their interconversions which lead to this mechanism were outlined in the previous project report; further experiments along essentially similar lines have strengthened the above interpretations.

Significance to Bio-medical Research and the Program of the Institute: Knowledge of mechanisms which control the interactions of proteins with nucleic acids and with other proteins is basic to understanding the properties of both normal and abnormal metabolic states. This experimental system offers potential insight into both of these areas of molecular interactions.

Proposed Course of Project: This project has been concluded, the results have been prepared for publication and recently appeared in the Journal of Biological Chemistry.

Publications:

Zimmerman, S. B. and Coleman, N. F.: Pancreatic Deoxyribonuclease: The Role of dimerization in the reversible thermal inactivation at acid pH. J. Biol. Chem. 246, 309-317 (1971).

Serial No. NIAMD-LMB-14
1. Molecular Biology
2. Organic Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Chemical and Structural Investigations of Nucleic Acids and Related Substances.

Previous Serial Number: NIAMD-LMB-2

Principal Investigator: H. Todd Miles

Other Investigators: Dr. F. B. Howard, Dr. Ishakawa, Dr. K. Ikeda, Dr. T. Lewis, and Mr. J. Frazier

Man Years
Total: 4 1/2
Professional: 3 1/2
Other: 1

Project Description:

Objectives: Basic chemical, biochemical, and structural studies of nucleic acids and related substances.

Methods: Infrared spectroscopy, nuclear magnetic resonance, spectroscopy, chemical synthesis and structure elucidation, optical rotation, ultraviolet spectroscopy, use of enzymes for synthesis and structure study.

Major findings: We have extended our previous investigation of the interaction of poly rT and poly r (U, T) copolymers with poly rA in detailed studies of the stoichiometry and cation dependence of complex formation. Below $[Na^+]$ = .04M a direct (2 ⟶ 1) dissociation of the two-stranded helix occurs when the solution is heated. At higher $[Na^+]$ a disproportionation reaction (2 ⟶ 3) occurs, followed by a second step at higher temperature (3 ⟶ 1).

We have sought to alter and control the relative stablities of two-and three-stranded helices by taking advantage of the higher charge densities of the latter. By the use of tetraalkylammonium salts rather than sodium as counterions we have achieved selective destabilization of the three-stranded helices (rA·2rT, rA·2rU and rA·2r (U,T) as compared to the corresponding two-stranded helices in these systems. The phase diagrams relating T_m and counterion concentration are radically changed by the use of these cations, which are less effective in screening electrostatic repulsion of the polynucleotide chains. The disproportionation (2 ⟶ 3) reaction is eliminated except at very high (> 0.7 M) cation concentration, and both 2 ⟶ 1 and 3 ⟶ 2 transitions are introduced into the region of moderate cation

concentration (~ 0.1 M) in neutral solution.

By using NEt_4^+ counterion in the studies of rA with the U,T copolymers we are now able to cause a simple 2 ⟶ 1 transition to occur throughout the range of copolymer composition. Like the 3 ⟶ 1 transition observed previously in Na^+, the dependence of T_m on rT content of the U,T copolymers (2 ⟶ 1) is linear. We conclude that a previous report of non-linear dependence of T_m on rT content of r(U,T) copolymers is erroneous. We have also examined the effect of the 5-methyl group in poly r (C,MeC) on T_m of complexes with poly I. The dependence is linear at all $[Na^+]$, confirming the absence of a proximity effect of the pyrimidine 5-methyl group.

Analogous copolymer studies have been carried out with U,BrU copolymers. The dependence of T_m (3 ⟶ 1) on BrU content of the complexes with poly rA is linear, showing the absence of a detectable proximity effect of the 5-bromo substituent, (cf. Howard et al., JBC, 1969).

2-N-dimethylaminoadenosine has been synthesized from the purine and tribenzoylribosyl chloride and converted by the cyanoethylphosphate procedure to the 5'-monophosphate. The nucleotide was used to prepare the 5'-pyrophosphate by the phosphomorpholidate method. The diphosphate was found to be a substrate for polynucleotide phosphorylase. The polynucleotide, poly 2-N Me_2A, forms an acid helical structure which is much more stable than that formed by poly A. The neutral polymer forms a stacked single stranded structure. The interaction with poly U is now being investigated.

We have obtained laser Raman spectra of good quality for several of the polynucleotide helices which we had observed previously in the infrared. The frequencies of the Raman and infrared bands should be the same, and we have found them to be so for the single-stranded polymers, though there are marked differences in relative intensities of the bands. In helical molecules, however, some of the bands show frequency differences which are well outside experimental error. The usual selection rules provide a basis for such discrepancies, and it appears probable to us that the explanation lies in helical selection rules derived by Higgs about 20 years ago.

We have observed uridine carbonyl band splitting in several polynucleotide helices and attribute the doublet to Fermi resonance, the coupling of an overtone or combination band with a fundamental. The splitting has been observed in two-stranded helices formed by poly U with substituted adenine nucleosides and also in alternating poly rA·U. Observation of dependence of Fermi resonance upon polynucleotide secondary structure is novel but can be rationalized in terms of solvent dependance observed in certain small molecules.

Isotopic derivatives (^{18}O, ^{2}H, ^{15}N) of 1-methyluracil have been prepared by specific synthesis which place the isotopes in known positions. Infrared and Raman spectra of these molecules have been observed in the solid state and in solution. The isotopic substitions permit reasonable assignments of the vibrational bands and definitive assignments of some.

Publications:

Ikeda, K., Frazier, J., and Miles, H. T.: J. Mol. Biol., 54, 59-84, 1970.

Miles, H. T.: Infrared spectroscopy of polynucleotide. In Cantoni, G., Davies, D. R. (Eds.): Procedures in Nucleic Acid Research . Vol. 2. in press.

Annual Report of the

Mathematical Research Branch

National Institute of Arthritis and Metabolic Diseases

Studies of the diffusion-consumption of oxygen at the capillary-tissue level have continued. The dynamics of extraction from the capillary network was analyzed for heterogeneous networks. An explicit formulation was obtained in terms of the product permeability x surface area. The problem of transient or nonsteady extraction of oxygen has been formulated in a general way which takes into account changes in blood flow, arterial oxygen content and oxygen consumption. A numerical procedure for solution of the problem has been devised and a computer program written. The program was used to analyze transient changes in oxygen extraction induced by transition from work to exercise. (Dr. J. M. Gonzalez-Fernandez and Mrs. S. Atta)

The long-term project concerned with constructing, developing and testing a group of interrelated models which provides a theory accounting for many varied sequences of events in a dendritic neuron has been continued. In particular illustrative examples of current injection to a single branch of a neuronal model composed of several extensive dendritic trees have been explored. The rigorous solution for dendritic input resistance, steady-state decrement of membrane potential and distortion of transient peaks provides new insight into neurophysiological problems which were previously vaguely formulated and poorly understood. Theoretical analysis of dendritic spines as receivers of synaptic input has continued. The distribution of types of spines (thin high resistance spines mostly distally, stubby ones proximally) has seemed paradoxical and has until now had no functional correlate or explanation. Mathematical results support the postulate that dendritic spines evolved as a "trade-off" device which sacrifices maximal synaptic power in order to gain refined control. (Dr. W. Rall with Mr. J. Rinzel, formerly DCRT-LAS)

The latest version of the SAAM computer system has been released consequent to conversion of the UNIVAC 1108 version for use on CDC and IBM machines. A delay element has been incorporated and the required restructuring will allow ready addition of other operational elements. (Dr. M. Berman and Mrs. M. Weiss)

A model for iodine kinetics in the Beagle under thyroid-blocked and unblocked conditions has been developed. (Dr. M. Berman with Drs. M. Barandes and B. Belshaw, Cornell Medical Center)

The effects of lithium on iodine kinetics in man is being studied. The results suggest that the single site of action of lithium is the hydrolysis of thyroglobulin. (Dr. M. Berman with Drs. R. Temple, J. Wolff and J. Robbins)

Study of free fatty acid-triglyceride kinetics has continued and previously developed models tested against new data. Incorporation of glycerol into triglyceride is similar to that of fatty acid but only a small

fraction is derived from plasma. (Dr. M. Berman with Drs. C. Malmendier and C. Delcroix, University of Brussels)

The method reported (W. London) lastyear for determining bounds on the number of intermediates in a straight-chain sequence of first order reactions has been extended. The method applies to any scheme of first order reactions with connectivity such that the shortest path from the observed species to the initially loaded species is p-1 where there are p species in the system. This type of connectivity is uniquely associated with an upper Hessenberg matrix. (Dr. W. London and Dr. J. Hearon)

If a product is formed from a precursor and the conversion is subjected to an inhibitor, the question arises whether the inhibitor acts on an obligatory intermediate or on a side product formed in a cul-de-sac reaction. For a fairly general class of systems this has been resolved by comparative analysis of the time course in the presence and absence of inhibitor, addition of inhibitor at various times after initiation of conversion and study of final yield as a function of inhibitor. When inhibition is known to occur by (ultimately) irreversible binding of an obligatory intermediate, a general expression for the final yield as a function of inhibitor has been derived and enables deductions regarding reaction steps subsequent to the point of inhibition. (Dr. W. London)

A mathematical model which describes recurrent epidemics of a disease has been developed. It is assumed that the population has a continuous input of susceptibles, that the disease has a definite incubation period and period of infectivity and produces permanent immunity. In particular measles (rubeola) is being considered and the data examined is on this disease. Plausible models usually yield damped periodicity leading to a stationary endemic level of such a disease. If the coefficient of infectivity is allowed to vary seasonally in a reasonable fashion, the model leads to periodicities with an endemic wave every two years or so. Analysis is being conducted to determine from data on measles, the infectivity coefficient as a function of time. (Dr. W. London and Dr. J. Yorke, University of Maryland)

Work has continued on the mathematical theory of renal function. There has, however, been a significant change in direction--away from computer simulation and toward analytical investigation. Useful approximate analytical models of both proximal tubular function and of the function of the medullary concentrating system have been developed. It has also been discovered that some of the systems of equations which have been used for computer simulation of the medullary counter flow system are inconsistent. Removal of the inconsistency can be achieved in various ways. Physiologically the most plausible is to include hydrostatic pressure as a driving force for the transmural movement of water from nephron to medullary capillaries.

The work on inconsistency was presented at the annual meeting of the Biophysics Society (New Orleans). A preliminary report of the work on proximal tubular function and the renal concentrating mechanism has been incorporated into a chapter "Elements of the Mathematical Theory of Renal Function", which is to appear in Engineering Principles in Physiology edited by J. H. U. Brown and a forthcoming publication of Academic Press. (Dr. J. Stephenson, NHLI, Associate Member, MRB)

The study of lumping in linear systems has continued. We cast the results in the language of compartmental analysis. It has long been known that if all compartments in an n-compartment system are expressible as a linear combination of some $m < n$ compartments, then each compartment is expressible in terms of at most m exponentials. It has now been shown that this is the case if and only if either a conditional degeneracy occurs or the matrix has repeated roots but is diagonable. The necessary and sufficient conditions for $k < m$ compartments to be expressible as in exponentials under all initial conditions has been derived. The conditions are the existence of M and K such that MA=KM where A is the compartmental matrix and $M=\text{diag}(I,M_o)$ with I the k-square identity. Such is the case if and only if the null space of M is an invariant subspace of A. (Dr. J. Hearon)

Serial No. NIAMD, ODIR, MRB-1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mathematics of kinetics and reaction-transport systems

Previous Serial Number: SAME

Principal Investigator: John Z. Hearon

Man Years:

Total:	1.3
Professional:	1
Others:	.3

Project Description:

Objectives: The objectives of this research remain as stated for calendar year 1962.

Methods Employed: General methods of matrix and vector space theory.

Major Findings: Since quite general and definitive results on lumping have been obtained, we devote this entire report to a summary of this study. All of this work detailed below was presented in an invited lecture at the SIAM National Meeting, Denver, Colorado, July, 1970.

A review of these reports will show that in 1964 a systematic study of lumping in linear systems was begun. While not a full time effort, this study has received some attention in each subsequent year.

Two important concepts were defined which were based on work reported in N. Y. Acad. Sci., 1963, and which have proved to be pivotal: Structural and Conditional Degeneracy (see project report 1964).

Innumerable special instances of lumping have been reported in the literature but the only other systematic investigations have been those of Mann and Gurpide. Following a lead in the N. Y. Acad. Sci. report, they showed (Bull. Math. Biophys., 31, 1969) that if each compartment in an n-compartment system is expressible as a linear combination of some $m < n$ compartments, then each compartment exhibits at most m exponentials. It has now been shown that in the case of distinct roots, (the only case they treat), such can occur if and only if we have conditional degeneracy; in general if and only if either conditional degeneracy or the matrix is

diagonable and derogatory.

In practice what is often observed is a linear combination of compartments and these averages or lumped entities may behave as single compartments. Suppose that the system in question obeys

$$\dot{x} = Ax$$

where A is an n-square matrix and x and n-vector. If M is m x n then the m-vector

$$y = Mx$$

has components which are linear combinations of those of x. If these linear combinations behave as compartments then we must have

$$\dot{y} = Ky$$

for some m-square matrix K. If this is the case we say that M lumps A and refer to y as the lumped vector. We now state the following completely general results on lumping:

1. The matrix M lumps A if and only if for some K we have

$$MA = KM$$

2. It is necessary and sufficient for M to lump A that the null space of M be an invariant subspace of A. Although this is the most abstract and deep-seated matrix-theoretic result on lumping to date it has, curiously enough, proved to be most useful and easily verified.

3. M lumps A if and only if for each pair of initial vectors $x_1(o)$ and $x_2(o)$ such that $Mx_1(o) = Mx_2(o)$ we have $Mx_1(t) = Mx_2(t)$ for all t.

4. If the null space of M is spanned by some subset of the eigenvectors of A, then M lumps A.

Utilizing these general results we now have the main theorem on lumping.

5. Compartments 1,2,...,k exhibit $m < n$ exponentials if and only if A is lumped by

$$M = \begin{pmatrix} I_k & 0 \\ 0 & M_1 \end{pmatrix}$$

where I_k is the k^{th} order identity.

6. If the system is lumpable as in 5 then there are an infinity of initial vectors which produce the same response in compartments 1,2,...,k.

7. In any symmetrizable case which is lumpable as in 5, there are realizable initial conditions such that every compartment is m exponentials.

Proposed Course: Work has been initiated which will form the basis for numerical studies of nearly lumped systems, i.e., systems which do not exactly meet the necessary conditions for lumping.

Serial No. NIAMD, ODIR, MRB-2
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mathematical formulation and analysis of problems relevant to experimental neurophysiology

Previous Serial Number: SAME

Principal Investigator: Wilfrid Rall

Man Years:

Total:	1.3
Professional:	1
Others:	.3

Project Description:

Over the past several years, this project has been concerned with constructing, developing and testing a group of interrelated mathematical models. This group of models provides a theory that can account for many different sequences of events in a dendritic neuron, and in certain populations of such neurons. Computational experiments performed with these models provide theoretical predictions that have been compared with the results of neurophysiological experiments and neuroanatomical observations. Also, insights have been obtained, which have led to new hypotheses, new interpretations, and to the design of new experiments.

1. Continued theoretical study of electric current injection to a single branch of a neuron model composed of several extensively branched dendritic trees. Although the basic theoretical results (formal mathematical solutions for steady state and transients) were obtained last year, considerable time and effort were devoted this year to the exploration of illustrative examples, and to preparing the first draft of what will be a comprehensive exposition and discussion of several interrelated results. Dendritic input resistance, steady state decrement of membrane potential throughout all dendritic branches, electrotonic distortion (attenuation and delay) of transient peak, are solved, illustrated and discussed, thus providing both rigorous solutions and new insight into neurophysiological problems that were previously vague and poorly understood. Many of these results were obtained in collaboration with John Rinzel (formerly of DCRT-IAS) who is now at the Courant Institute of Mathematical Sciences, New York University.

2. Dendritic spines: continued theoretical analysis and exploration of the functional significance of dendritic spines as receivers of synaptic input. Recent electron microscopic studies have shown (a) that pyramidal cells of mammalian neocortex receive most of their synaptic contacts upon their dendritic spines, and (b) that spines having long thin stems (high resistance) occur with highest frequency on the more distal dendritic branches, while the more stubby spines occur with highest frequency on dendritic trunks and proximal dendritic branches; this was learned from correspondence and from papers published in 1969 and 1970. To the few who know these facts, they seem paradoxical and they beg functional interpretation. The mathematical results obtained last year (with John Rinzel) are being written up to show how they provide a basis for resolving some of these paradoxes. We develop and explain our postulate that dendritic spines may have evolved as a device which sacrifices maximal synaptic power in exchange for refined control of relative synaptic potency; this could contribute to the plasticity of the nervous system.

3. Conducted an NIH Tutorial Seminar on the subject of Neuronal Dendritic Branching. I prepared the material for seven 1-1/2 hour seminars. Considerable useful discussion was generated.

4. Time and effort were given to a number of informal consultations and to referee theoretical manuscripts for several journals.

Advisory Councils:

Central Council of IBRO (International Brain Research Organization, affiliated with UNESCO).

Committee on Brain Sciences, National Research Council, National Academy of Sciences.

National Council of the Society for Neuroscience.

Publications:

Rall, W.: Cable properties of dendrites and effects of synaptic location. In Andersen, P. and Jansen, J. K. S. (Eds.): Excitatory Synaptic Mechanisms. Oslo, Norway, Universitetsforlaget, 1970, pp. 175-187.

Rall, W.: Dendritic neuron theory and dendrodendritic synapses in a simple cortical system. In Schmitt, F. O. (Ed.): The Neurosciences: Second Study Program. New York, Rockefeller University Press, 1970, pp. 552-565.

Serial No. NIAMD, ODIR, MRB-3
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mathematical description of the transport-chemical reaction kinetics of substances in the blood-capillary-tissue complex

Previous Serial Number: SAME

Principal Investigator: Jose M. Gonzalez-Fernandez

Other Investigators: Susie E. Atta

Man Years:

Total:	2.3
Professional:	2
Others:	.3

Project Description:

Objectives: The broad objectives of this research remain substantially the same as the ones stated in the previous reports.

Major Findings:

Steady state:

1. The study of the diffusion consumption of oxygen in regular polygons as outlined in the previous report was completed. A paper containing the results was submitted for publication.

2. The dynamics of substrate extraction from the capillary network was analyzed for heterogeneous capillary networks. An explicit formulation of extraction in terms of capillary permeability x capillary surface and flow patterns was obtained extending Renkin's results (Renkin, E.: Normal regulation of tissue circulation. In Price, H. L. and Cohen, P. J. (Eds.): Effects of Anesthetics on the Circulation. Springfield, Ill., Charles C. Thomas, 1964, pp. 171-181). The flow patterns that optimize the substrate extraction were computed.

Nonsteady state:

1. A general formulation for the nonsteady state extraction of oxygen due to changes in blood flow, in arterial oxygen content and oxygen

consumption was formulated. A procedure to solve the problem numerically was developed; a computer program was written.

2. The experimental results of Stainsby and Otis (Stainsby, W. and Otis, A. B.: Blood flow, blood oxygen tension, oxygen uptake, and oxygen transport in skeletal muscle. Am. J. Physiol. 206: 858-866, 1964) during transient changes in oxygen extraction from rest to exercise were examined numerically with the aid of the computer program above mentioned.

Serial No. NIAMD, ODIR, MRB 4.1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Analysis of kinetic data and modeling

Previous Serial Number: SAME

Principal Investigator: Mones Berman

Other Investigators: Drs. C. Malmendier, C. Delcroix (Univ. of Brussels); R. Levy, D. Bilheimer and S. Eisenberg (NHLI); R. Andres and group (NICHD)

Man Years:

Total:	1
Professional:	1
Others:	0

Project Description:

Objectives: Analysis of data on metabolic systems, and the further development of modeling techniques through the use of the SAAM computer program.

Major Findings: The modeling of FFA-triglyceride kinetics in man (normals and abnormals) was continued in collaboration with Drs. Malmendier and Delcroix of Brussels. The models previously developed by our group were tested against the new data and found to be consistent. A combination of our models A and C (J. Clin. Invest., 1970, 2298) was most appropriate: The FFA subsystem required three compartments since recycling of triglyceride fatty acids could not account for the slowest FFA component.

The incorporation of plasma glycerol into TG was also modelled and found to be very similar to plasma FFA incorporation. This suggests that glycerol and FFA combine early in the formation path of TG. The amount of glycerol utilized from plasma for TG was a small fraction of the total required--contrary to FFA utilization.

A further finding was the fact that short FFA chains which recycle from the direct oxidative path to CO_2 are not utilized for TGFA.

The modeling of the metabolism of VLDL is in progress in collaboration with Dr. R. Levy, D. Bilheimer and S. Eisenberg of the NHLI.

Modeling of the Glucose-Insulin system is also in progress in collaboration with Dr. Andres and his group (NICHD).

Publications:

Shames, D. M., Frank, A., Steinberg, D., and Berman, M.: Transport of plasma free fatty acids and triglycerides in man: a theoretical analysis. J. Clin. Invest. 49: 2298-2314, 1970.

Quarfordt, S. H., Frank, A., Shames, D. M., Berman, M., and Steinberg, D.: Very low density lipoprotein triglyceride transport in type IV hyperlipoproteinemia and the effects of carbohydrate-rich diets. J. Clin. Invest. 49: 2281-2297, 1970.

Phang, J. M., Finerman, G. A. M., Singh, B., Rosenberg, L. E., and Berman, M.: Compartmental analysis of collagen synthesis in fetal rat calvaria. I. Perturbations of proline transport. Biochim. Biophys. Acta 230: 146-159, 1971.

Shames, D. M., Berman, M., and Segal, S.: Effects of thyroid disease on glucose oxidative metabolism in man. A compartmental model analysis. J. Clin. Invest. 50: 627-641, 1971.

Serial No. NIAMD, ODIR, MRB 4.2
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Iodine kinetics

Previous Serial Number: SAME

Principal Investigator: Mones Berman

Collaborating Investigators: M. Barandes, B. Belshaw (Cornell Medical Center, New York); R. Temple, J. Wolff and J. Robbins (NIAMD)

Man Years:

Total:	1
Professional:	1
Others:	0

Project Description:

Objective: To develop a model for the kinetics of iodine and thyroid hormones in man.

Major Findings: A model for the kinetics of iodine in the Beagle under thyroid-blocked and unblocked conditions was developed. Under both conditions I^-, T_3 and T_4 kinetics were studied. The derived model is quite similar to that for man although there are differences, especially in the metabolism of T_4. (Barandes, Belshaw, Berman)

The effects of Lithium on the kinetics of iodine was modelled in collaboration with Dr. Temple, Wolff, and Robbins. Although this work is still in progress, the results suggest that the rate of hydrolysis of thyroglobulin is the only variable affected by Li. Modeling of Li effects also sheds light on the intrathyroidal iodide release pathways in hyperthyroidism and aids in the further definition of the general model.

Publications:

Koutras, D. A., Berman, M., Sfontouris, J., Rigopoulos, G. A., Koukoulommati, A. S., and Malamos, B.: Endemic goiter in Greece: Thyroid hormone kinetics. J. Clin. Endocrinol. Metab. 30: 479-487, 1970.

Serial No. NIAMD, ODIR, MRB 4.3
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: SAAM Computer System

Previous Serial Number: SAME

Principal Investigator: Mones Berman

Other Investigators: Marjory F. Weiss

Man Years:

Total:	1.3
Professional:	1.3
Others:	0

Project Description:

Objectives: To develop a general purpose computer program for modeling bio-kinetic systems that may readily be used by investigators not sophisticated in mathematics or programming. The SAAM program was initially developed in 1959 and various phases have been developed and added to it since.

Major Findings: The latest version, SAAM25, has now been released for use to other computer centers. Considerable efforts were devoted to the conversion of the UNIVAC 1108 version for use on CDC and IBM machines.

A new operational element--a delay--is now being tested in SAAM. This element acts like a delay line having a specified delay time and resolution. The delay element bypasses the Runge-Kutta or other differential equation solution techniques and operates quite efficiently through a special indexing technique.

A major restructuring of the computational logic of SAAM was made to accommodate the delay element. The new computational structure also permits the addition of other operational elements (planned for the future) and results in more efficient solutions of problems.

Progress on a conversational SAAM system has been slow but is continuing. The selection of an appropriate cathode ray display terminal is under consideration.

Serial No. NIAMD, ODIR, MRB-5
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mathematical analysis of kinetic problems in biochemistry

Previous Serial Number: SAME

Principal Investigator: Wayne P. London

Man Years:

Total:	.5
Professional:	.5
Others:	0

Project Description:

Objectives: To develop mathematical models of the kinetics of biochemical reactions.

Major Findings: The methods for obtaining the maximum and minimum number of precursors in a sequence of first order reactions (described last year) were shown to apply any scheme of first order reactions in which the shortest path from X_1 to X_p is of length p-1. Properties of the class of matrices that correspond to such compartmental systems were investigated.

Questions arising from the study of kinetics of the conversion of prothrombin to thrombin [begun by Drs. J. Z. Hearon and N. R. Shulman (NIAMD-CH)] were studied:

(1) For the scheme $P \rightarrow X \begin{matrix} \nearrow \cdots \rightarrow T \\ \searrow \cdots \rightarrow C \end{matrix}$, the final yield of T is given by $T(\infty) = N/(1+\alpha)$ where N is the normal yield of T when the path to C is absent, and α is a complicated function of the rate constants. The functional form of α was related to the nature of the reactions between X and T and between X and C; thus data on the final yield of T provides information about reactions occurring after the intermediate X.

(2) For a scheme of reactions $P \rightarrow X \underset{\downarrow S}{\rightleftharpoons} (Y_i) \rightarrow T$ it was shown if S is added at different times after the reaction is begun, the ratio of the amount of additional T formed in the presence of S to the additional amount of T formed in the absence of S is a temporal constant if and only if

there are no intermediates between X and T.

(3) It was also shown why the model $Y \rightleftharpoons P \rightarrow T$, is inconsistent with the kinetic data of the conversion of prothrombin (P) to thrombin (T) in the presence of soybean trypsin inhibitor (S).

The analysis of the kinetics of enzyme reactions in which a modifier combines with the enzyme and a substrate (London and Steck, 1969) was applied to the calcium-myosin ATP'ase system in consultation with Dr. R. Avena and the late Dr. W. Bowen (NIAMD-LBC). A reaction model was proposed in which CaATP is the substrate for native myosin A and two moles of ATP inhibit the reaction (Avena, R. M. and Bowen, W. J.: Adenosine Triphosphate Inhibition in Myosin Adenosine Triphosphate Systems. J. Biol. Chem. 246: 2265-2270, 1971).

Serial No. NIAMD, ODIR, MRB 5.1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mathematical analysis of the dynamics of infectious diseases

Previous Serial Number: NONE

Principal Investigator: Wayne P. London

Cooperating Units: Professor James A. Yorke
Institute for Fluid Dynamics and Applied Mathematics
University of Maryland

Man Years:

Total:	.5
Professional:	.5
Others:	0

Project Description

Objectives: To develop mathematical models of the dynamics of infectious diseases in large populations.

Major Findings: A mathematical model that describes the recurrent epidemics of measles (rubeola) has been developed. The model is a system of two nonlinear ordinary delay differential equations,

$$\dot{x}(t) = -\beta x(t)y(t) + \gamma(t)$$

$$\dot{y}(t) = \beta[x(t-a)y(t-a) - x(t-(a+b))y(t-(a+b))]$$

where $x(t)$ is the number of susceptibles; $y(t)$, the number of infectives; $\gamma(t)$, the birth rate of susceptibles; a is the incubation period; b, the period of infectivity, and β, the coefficient of infectivity. The model assumes that the rate at which people become infectious is directly proportional to the number of susceptibles, the number of infectives, and the coefficient of infectivity, β. The delays enter because people who became infectious at time t were exposed at time $t-a$, and people who lose their infectivity at time t were exposed at time $t-a-b$.

The linearized version of the equations has been studied. If the delays

are assumed to be small relative to the basic time scale (10-17 days versus 10-15 years), the equations are those of a harmonic oscillator, the period of which equals the geometric mean of incubation period times the mean time before a susceptible becomes infectious.

The full model has been studied numerically on a computer as a system of two difference equations. If the coefficient of infectivity β is fixed throughout all months of the year, the solution is a series of damped oscillations that asymptotically approach a constant, that is, the disease approaches a constant, endemic level in the population. If the coefficient of infectivity is decreased by 50% during three months of the year, to simulate the decline of measles cases reported during the summer months, the solution is periodic with an epidemic wave occurring every two years. If β is held constant throughout the year, but the entry of susceptibles into the population is seasonally adjusted, to simulate the gathering of children in school in the fall, the solution is again periodic with an epidemic every two years. In large urban areas such as New York City, prior to the introduction of the measles vaccine, measles epidemics occurred every other year.

We have recently obtained from the New York City Health Department the reported cases of measles by month in that city during the past forty-five years. We are currently using these data to test the validity of the model and to calculate the seasonal and yearly variation in the coefficient of infectivity.

Proposed Course: We shall continue to use differential delay equations to model the dynamics of infectious diseases in large populations. We plan to study diseases such as rubella, influenza, hepatitis, and cholera, where the epidemiology is more complicated than with measles.

ANNUAL REPORT OF CLINICAL INVESTIGATIONS, NIAMD

July 1, 1970 to June 30, 1971

Dr. Robert S. Gordon, Jr., Clinical Director

- - -

Fiscal year 1971 saw the addition of a new clinical research section to those previously active in NIAMD; this is the unit attached to the Phoenix Indian Medical Center, plans for which were mentioned in last year's report. Dr. Scott Grundy entered on duty January 2, 1971 as Section Chief and head of the clinical service, and the first patients were admitted shortly thereafter. At this time, he is being assisted by 2 Clinical Associates, 2 secretaries, 2 technicians, 1 laboratory aid, a research nurse, and a dietician. Initial studies being undertaken by the new section center on the etiology and pathophysiology of cholesterol gallstone disease, to which the American Indian is especially prone; it is anticipated that with added personnel and other facilities to be provided during the coming year, studies related to diabetes and its complications may also be initiated. The scientific program of the Phoenix Clinical Research Section will be described in more detail in next year's report, by which time specific projects will have taken form, and when initial results may be expected to have been obtained.

The clinical services of NIAMD in Bethesda carried the following patient load during the period April 1, 1970 through March 31, 1971: 409 patients were admitted to the Clinical Center, for a total of 15,202 patient days in hospital. The average length of hospitalization was 37 days, and our average bed occupancy was 61%. During the same period, 2,329 outpatient visits were paid to the clinics of this Institute.

Beginning with the first of April, 1970, a computerized file of inpatient admissions has been developed. This is based simply on the capture and retention of information used in preparing the agenda for Dr. Gordon's weekly rounds with the Clinical Associates, at which new admissions are introduced to the group, and their medical problems summarized. The file makes it possible to retrieve very quickly the name of any patient whose chart number is known, or vice versa. It may also be searched on the basis of terms used in the admitting diagnosis. The following data were collected for the one-year period April 1970 through March 1971:

Admissions by Nursing Unit

9 East	143
9 West	143
8 West	123
Total	409

Admissions by Branch		Assigned Beds	Admissions per Bed	Clinical Associates on Duty
ARB	100	15	6.7	8
DHDB	46	11	4.2	2
CEB	100	16	6.3	5
CHB	42	9	4.7	2
MDB	61	10	6.1	3
PMB	60	7	8.6	2
Total	409	68	6.0	22

Admissions by Initial Diagnosis

Acromegaly	26
Cystic fibrosis	49
Diabetes mellitus	13
Hemophilia	8
Hyperparathyroidism	19
Hyperthyroidism	10
Hypoglycemia	7
Idiopathic thrombocytopenic purpura	15
Malabsorption	14
Normal volunteer	49
Osteoporosis	7
Rheumatoid arthritis	18
Sjögren's syndrome	25
Systemic lupus erythematosus	37
All others	112
Total	409

There were no changes in the senior staff of NIAMD clinical research branches during FY 1971. Dr. Laster has continued throughout the year in his assignment to the White House science advisory staff, but expects to rejoin the Institute during the summer of 1971. Dr. Paul Plotz, a former Clinical Associate, returned to assume a permanent position with ARB, from which Dr. Norman Talal, a mainstay for almost a decade, will depart in June to assume a new post as Associate Professor of Medicine and Microbiology, Univ. of California in San Francisco. and as Chief, Division of Clinical Immunology and Arthritis. Veterans Administration. During 1971 the permanent staff of NIAMD has been assisted by 22 Clinical Associates. 5 Visiting Scientists, and 26 Guest Workers. The latter two groups represented 7 foreign countries.

1. Articular Disease

The chronic polyarthritis of swine induced by experimental infection with Mycoplasma hyorhinis has been extended to 588 days. Evidence of active disease in the joints is histologically apparent long after antibodies to the organism have virtually disappeared from the serum implying that the inflammation persists due, not to the organisms, but to self-perpetuating processes initiated by the organism. The potential similarities to rheumatoid arthritis are apparent. Tissue studies for immunoglobulin and collagenase are underway with immunofluorescent methods. (Decker, Barden (not NIH), Hopps (DBS), Dalgard (not NIH), Ennis and Johnson (I)).

The assessment of the merits of prophylactic synovectomy continues. (Decker and Peterson (not NIH)). The studies of physiologic variables about inflamed joints has been completed and is in press. Lactate production of the inflamed synovia was found to be 10 times that of normal joints. ^{133}Xenon washouts proved to be an excellent measure of inflammation as graded clinically or histologically. Post-synovectomy blood flow and O_2 consumption continued elevated but lactate production was greatly reduced. Only O_2 consumption did not change toward normal upon intra-articular corticosteroid injection. (Goetzl, Decker, Falchuk (H), Zeiger (NM) and Adams (not NIH)).

2. Sjogren's Syndrome

Salivary scintigraphy with ^{99m}technetium has proven to be extremely useful in assessing salivary gland disfunction. Striking improvement has been observed upon treatment with adrenal corticosteroids or cyclophosphamide. The measure appears to relate more closely to disease activity than do any of the other non-invasive techniques used. (Talal, Tarpley (D), Schall (CC) Asofsky (I), Lightbody (D), Anderson and Cummings (D)).

3. Systemic Lupus Erythematosus (SLE)

Short-term in-hospital treatment of SLE nephritis with cyclophosphamide (7 patients) or placebo (6 patients) has been evaluated and the results are in press. The drug has a clearly favorable effect on serum complement and DNA-binding antibody levels, some effect on urinary sediment, proteinuria and extra-renal manifestations of SLE, and little or none on creatinine clearance at least in the short term. Unfavorable effects, such as alopecia and cystitis, were numerous but generally tolerable. The study provided evidence of a relationship between dosage and response; when low dosage was enforced by marrow sensitivity as expressed by leukopenia, a favorable change in measures was less likely to occur. (Decker, Talal, Kaltreider (not NIH), Staples (not NIH), Steinberg and Goetzl (not NIH)). Azathioprine is now being studied in comparison with randomly assigned cyclophosphamide and placebo. Eighteen patients have completed the study or are in process. No conclusions are yet possible but we have observed a hypersensitivity reaction to azathioprine (rash, malaise, fever and abnormal liver chemistries),

a picture not yet noted in a much larger group treated with cyclophosphamide. (Decker, Steinberg, Hadler and Salisbury).

Fibrin split products are found in the urine of SLE patients with active nephritis. Thus far, we are satisfied that the amounts present are not exclusively a function of proteinuria and, thus that their meaning and relationships are worthy of continuing study. (Decker, Steinberg, Gralnick (CC) and Marchesi (CC).

Efforts to understand SLE as due to a mixture of genetic and viral factors have continued using primarily assays for nucleic acid binding antibodies which have been applied to both human (Talal, Steinberg and Schur (not NIH) and murine SLE. (Talal, Chused, Jacobs, Steinberg, Gazdar (C), Carpenter (BS) and Schur (not NIH)). In the latter process, it has been shown that cells making anti-DNA antibody are located in the spleen of the New Zealand Black/White (B/W) hybrid. The antibodies can be found in the serum of heavily irradiated syngeneic recipients of a spleen cell population taken from a DNA-binding donor. Thymus and bone marrow transfers do not result in antibody production.

Moloney leukemia virus accelerates the spontaneous production of anti-RNA antibodies by B/W mice. This increased production can be prevented by pretreatment with polyinosinic·polycytidylic acid and cyclophosphamide. Tolerance is then said to exist. It seems possible that tolerance induction of this type might prevent the development of anti-DNA antibodies with their potential for causing SLE nephritis. (Talal, Chused, Jacobs, Steinberg, Gazdar (C), Carpenter (BS) and Schur (not NIH)).

4. Immunoglobulin Structure and Function

Further studies of the affinity labeled binding site of a mouse myeloma protein have been carried out with cross-linking reagents reacting with the free amino group of 3 aminotyrosine, itself generated from a specific tyrosine residue by reducing the azotyrosine bond induced by the diazonium affinity labeling compound. The method provides information on the structural relationships between the tyrosine residues and other residues to which cross-linking occurs. (Metzger, Goetzl and Hadler). Tetranitromethane specifically introduces nitro groups into the 3 position of tyrosine residues. Since these residues play an important role in binding siges and sin-e the nitro groups can be selectively labeled via 3 aminotyrosine, as mentioned above, studies on the effect of ligand in protecting against the action of tetranitromethane are in progress. (Metzger, Otchin and Lee).

Human gamma E has been detected on the surface of basophils but not on other white cells. Burro antibody to human gamma E was reacted with the cells which were then "counterstained" with a hybrid rabbit antibody capable of reacting with the burro IgG and horse ferritin. The ferritin was identifiable by electron microscopy. (Metzger, Sullivan and Grimley (C).

Since several mouse myeloma proteins are able to bind phosphoryl choline, reagents suitable for purifying and affinity labeling of the binding sites have been developed. P-azophenylphosphoryl choline has proven useful as an immune adsorbent and as a labeling reagent. The work continues but labeling has been found on the light chains of 2 antibodies and on the heavy chains of 2 others. (Metzger and Chesebro).

Studies of antigen receptor sites on immune cells are beginning with efforts to enrich cells bearing such receptors. Cells which stick to antigen-labeled polymer beads can be recovered and shown to be immunocompetent. (Plotz and Salisbury).

5. Biochemical Pharmacology

The pathways by which compounds in the gut lumen are catalyzed have been attributed to the action of the intestinal microbiota. It was therefore surprising to find that not a single organism of many isolated from feces was capable of the complete degradation of caffeic acid. Several organisms were capable of individual steps in the process but one of the final steps went forward only with a mixed culture of two organisms. The methods involved appear useful for assessing the production of various micriobial metabolites which are absorbed and do contribute to pathologic states such as uremia. (Goldman, Peppercorn and Milstien).

High resolution analysis of urinary components has been begun using a defined synthetic diet in order to simplify the number of ultraviolet-absorbing and carbohydrate-reactive components found. By the fifth day on the diet urinary components seem to have reached a steady state with a great reduction in the number of compounds found. (Goldman and Young (CC)).

Because of the value of fluorinated analogues in establishing a molecular basis for biological phenomena, a detailed study has been made of the biochemical properties of γ-fluoroglutamate. The 4 isomers formed by organic synthesis were separated by column chromatography and enzymatic resolution. The configurations at the α-carbon of these isomers were related to those of glutamate by the action of specific enzymes. In addition the relative configurations of the two asymmetrical carbon atoms has been established. Enzymes reactive with γ-fluoroglutamate include glutamate decarboxylase, carboxypeptidase G, glutamine synthetase and D-glutamate cyclase. A small amount of the analogue can be incorporated into peptide linkage by growing E. coli.

Serial No. NIAMD-ARB-1c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Role of Infection in Rheumatoid Diseases

Previous Serial Number: NIAMD-ARB-3c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Jerri Barden (Hazleton Laboratories), Dr. Hope Hopps (DBS-LVI), Dr. Dan W. Dalgard (Hazleton Laboratories), Dr. Robert S. Ennis and Dr. John Johnson (I-LCI).

Cooperating Units: Hazleton Laboratories, Division of Biologic Standards, Laboratory of Viral Immunology; National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigations.

Man Years:

Total:	1.5
Professional:	1.0
Other:	.5

Project Description:

Objectives:

The overall subject is to discover the cause of rheumatoid arthritis. To this end we are working with animal mycoplasmal arthritides.

Methods Employed:

Half of a group of 30 young specific-pathogen-free swine were given an intraperitoneal innoculation of Mycoplasma hyorhinis and kept isolated from the saline innoculated controls. Intensive clinical, microbiological, immunological and histopathological studies of the infected and normal animals were carried out. Swine synovia from both groups have been grown in tissue culture without antibiotics.

In addition, a second group of 36 Yorkshire swine was introduced in a second study in July 1969. Of these animals, 12 were kept as controls, and the remaining 24 were innoculated with Mycoplasma hyorhinis.

Similar clinical, microbiological, immunologic and histopathologic studies are being carried out, and in addition, attempts are being made to identify M. hyorhinis antigen or organism in the late, chronic phases by using immunofluorescent techniques.

Major Findings:

Study I

A febrile polyarthritis and polyserositis developed within two weeks of innoculation. The organism was readily recovered from many tissues.

The titer of M. hyorhinis metabolic inhibiting antibody rose steadily into the fifth month of the study. Chronic arthritis was seen clinically in the infected Yorkshire but not in the infected miniature swine.

Histopathologic study revealed that the disease appeared to be divided into three stages: an acute stage which lasts from the first to third weeks, and is characterized by an acute synovitis, predominantly polymorphonuclear exudate, interstitial edema and hyperemia, and diffuse mononuclear infiltrate.

The subacute and second stage (3 weeks - 3 months) shows disappearance of the exudate, occurrence of perivascular infiltrates of lymphocytes, plasmacytes and mononuclear cells, and the beginning of articular cartilage damage.

The third or chronic stage is composed of animals sacrificed from 90 days - 254 days. There is a general decrease in symptoms of systemic disease. Chronic arthritis bearing many resemblances to human rheumatoid arthritis is seen including synovial hypertrophy and hyperplasia, lymphoid nodule formation, and deposits of a fibrinoid-like material in the synovial villi. In this last stage organism was recovered from only 2/16 joints, in comparison with 16/19 joints in Stage I and 17/23 joints in Stage II.

Study II

To date, all animals in the second study have been sacrificed at time intervals ranging from 20 to 588 days post infection.

Microbiologic and histopathologic findings have paralleled the first study, except that animals sacrificed in the chronic stage appear to be undergoing a significant attempt at reparation of joint damage.

Attempts to demonstrate continuing immune response have included the following:

a. Skin testing of late stage animals with specific components of M. hyorhinis organism.

b. Lymphocyte transformation of cells from late stage animals against M. hyorhinis antigen.

c. Immunofluorescent staining of specially prepared cold paraffin embedded specimens of synovium and lymph nodes taken from control and infected animals at the time of sacrifice. Staining is performed with rabbit-anti pig γA, γM and γG in order to demonstrate the presence of immunoglobulin producing cells.

In addition, specimens of cartilage are being analyzed by Dr. M. Friedland for the presence of collagenase.

Proposed Course:

Statistical analysis of histopathologic material similar to that done in Study I. Analysis of immunofluorescence data in the light of possible auto-immune processes.

Significance to Bio-Medical Research and the Program of the Institute:

The above late phase findings may be interpreted as those of persisting, masked infection or of chronically active disease initially triggered by infection but persisting as "auto-immune" disease.

Honors and Awards: None

Publications:

1. Mycoplasma hyorhinis swine arthritis. I. Clinical and microbiological features. J. A. Barden and J. L. Decker. Arthritis Rheum (In press).

2. Mycoplasma hyorhinis swine arthritis. II. Morphological features. R. S. Ennis, D. Dalgard, J. Barden and J. L. Decker. Arthritis Rheum (In press).

Serial No. NIAMD-ARB-2c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Swine Gamma Globulins

Previous Serial Number: NIAMD-ARB-9C

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. John S. Johnson (I-LCI), Dr. H. Benfer Kaltreider (Univ. of California at San Francisco) and Dr. Jerri Barden (Hazleton Laboratories).

Cooperating Units: National Institute of Allergy and Infectious Diseases, University of California at San Francisco and Hazleton Laboratories, Falls Church, Virginia

Man Years:

Total:	.5
Professional:	.3
Other:	.3

Project Description:

Manuscript preparation on this material is in its final stages.

Further work with swine immunoglobulins is proceeding with immunofluorescent methods - See Serial No. NIAMD-ARB-1c.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-3c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Prophylactic Synovectomy in Rheumatoid Arthritis

Previous Serial Number: NIAMD-ARB-2c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Leonard T. Peterson, Orthopedic Consultant, George Washington University School of Medicine

Cooperating Units: George Washington University School of Medicine, Washington, D. C.

Man Years:

Total:	.5
Professional:	.3
Other:	.3

Project Description:

Objectives:

To discover whether or not prophylactic synovectomy, as defined below, will alter the functional or structural prognosis in rheumatoid arthritis.

Methods Employed:

Patients with rheumatoid arthritis who have had at least six months of active inflammation in both of a pair of symmetrical joints, neither of which show radiological changes other than osteoporosis, have been admitted to the study. The disease must be sufficiently symmetrical to have all parties -- the investigator, the surgeon and the patient -- agree to have either one of the pair operated. The side to be done is selected by lot.

The patients are then seen at intervals of six months, one year, and two years. On these visits they undergo standard evaluation for function and radiological examination.

Major Findings:

Thus far it is possible to say that, at 18 months followup, the operated knee was still preferred clinically. The radiological changes have not been impressive in either knee.

Significance to Bio-Medical Research and the Program of the Institute:

There has been essentially no controlled work on this important therapeutic modality which is being widely urged on the basis of inadequate data. There is reason to believe that, almost alone among treatment methods, it will prevent joint damage. This needs to be established.

Proposed Course:

Erosive disease at the knee is difficult to assess radiologically; extensive disease can go undetected.

A study similar to the above can be done on the metacarpophalangeal (MCP) joints which are easier of assessment and more frequently symmetrically involved in rheumatoid arthritis. The study would have been done on MCP joints except for the unavailability of an expert hand surgeon. We are now discussing such a study with Dr. John Adams, Professor and Chairman, Department of Orthopedics, George Washington University. Dr. Adams is interested and hopes to be available for work here in the Clinical Center one day a week commencing in September 1971.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-4c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Assessment of Synovial Physiologic Variables

Previous Serial Number: NIAMD-ARB-1C

Principal Investigator: Dr. Edward J. Goetzl

Other Investigators: Dr. John L. Decker, Dr. Kenneth H. Falchuk (H:LKEM), Dr. Louis S. Zeiger (Nuclear Medicine, NIH), and Dr. John P. Adams (George Washington University).

Cooperating Units: National Heart Institute, Laboratory of Kidney and Electrolyte Metabolism, Nuclear Medicine, NIH, and George Washington University School of Medicine, Department of Orthopedic Surgery.

Man Years:

Total:	.5
Professional:	.3
Other:	.3

Project Description:

Objectives:

The primary objective is to relate synovial tissue metabolic rate and circulatory flow with the histologic evaluation and the clinical and laboratory assessment of disease in joints with various arthropathies.

Secondary purposes are: 1) to provide *in vivo* corroboration of the high rates of oxygen utilization and carbon dioxide and lactic acid production by rheumatoid synovial tissue seen *in vitro*, 2) to explore methods of applying isotopic wash-out to the measurement of regional synovial blood flow, 3) to determine the relative quantitative roles of tissue metabolism and regional circulation in establishing the steady state PO_2, PCO_2 and pH in synovial fluid, and 4) to evaluate the ability of various therapeutic agents to influence the nature and the rate of metabolism of synovial tissue.

Methods Employed:

Patients were selected who have rheumatoid arthritis, other inflammatory arthritides, traumatic or degenerative joint disease. They had a complete

clinical, radiologic, and laboratory evaluation prior to the actual study.

All synovial fluid was aspirated from single knee joints. Radioactive xenon (^{133}Xe) in saline, at a dose of 50-100 μCi was injected into the knee with rapid mixing of the contents of the joint. The rate of fall of radioactivity in the knee joint was monitored externally and plotted on semi-logarithmic paper. These curves were analyzed by computer based on a two-compartment model for regional blood flow. The half-time for the fall of radioactivity in the earliest definable component is considered proportional to synovial blood flow.

These same knee joints, either with large effusions or after saline instillation, were allowed to equilibrate with the patient breathing 40-50% oxygen. The joint was isolated from its circulation by an arterial tourniquet, and the rate of fall of PO_2 and pH and the rate of rise of PCO_2 and lactic acid in synovial fluid was followed using timed serial aspirations of fluid. All joints were biopsied. In some patient, the studies were repeated after either synovectomy or a major alteration in the therapeutic program.

Major Findings:

There was a 2-3 fold higher oxygen uptake rate and a 10-12 fold higher lactate production rate in the severe rheumatoid joint washouts as compared to control joints.

^{133}Xe wash-out from the intra-articular space was shown to be due to circulation in the knee joint as a whole and the results of this evaluation showed a 3 fold greater mean wash-out rate from the rheumatoid joints (48 studies) than control joints (7 studies) with extensive overlap between the two groups. The metabolic variables correlated with tissue metabolic demand as graded in synovial biopsies. ^{133}Xe wash-out rate correlated with knee joint inflammation estimated both clinically and histologically.

After synovectomy in four patients, the operated knee showed a lower lactate production than the opposite knee in the three patients with a favorable result. Neither synovial oxygen uptake nor ^{133}Xe wash-out rate changed significantly. Intra-articular corticosteroid injection (8 patients) resulted in decreased lactate production and a decreased ^{133}Xe wash-out rate in the injected knee and variable results in the untreated knee. Oxygen consumption again was unchanged after therapy.

Significance to Bio-Medical Research and the Program of the Institute:

These metabolic measurements reflect a vector of rheumatoid arthritis which cannot be evaluated in any other way. This proliferative vector is largely responsible for the destruction of joints seen in advanced arthritis. Regional blood flow is an objective measure of both tissue proliferation and local inflammation in the joint. Therefore, observations on the effect of various

therapeutic maneuvers on these components of disease will prove invaluable in attempting to halt joint damage at an early stage.

Proposed Course:

The study will be considered terminated upon publication of the results. The report of the work has been accepted for publication in The Journal of Clinical Investigation.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-5c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Clinical and Immunological Studies in Sjogren's Syndrome

Previous Serial Number: NIAMD-ARB-4C

Principal Investigator: Dr. Norman Talal

Other Investigators: Dr. Thomas A. Tarpley (D-OMS), Dr. Gerald L. Schall (CC-NM), Dr. Richard Asofsky (I-LMI), Dr. Philip Lightbody (D-DS), Dr. Larry Anderson and Dr. Norman Cummings (D-OMS)

Cooperating Units: National Institute of Dental Research, Dental Services Branch, Oral Medicine and Surgery Branch; Clinical Center, Department of Nuclear Medicine; National Institute of Allergy and Infectious Diseases, Laboratory of Microbial Immunity

Man Years:

Total:	.5
Professional:	.3
Other:	.3

Project Description:

Objectives:

To understand the pathogenesis of Sjogren's syndrome and its variants, with particular emphasis on immunologic abnormalities and the nature of the salivary gland infiltrates.

Methods Employed:

1. Biopsy of minor salivary glands in lower lip for studies of immunoglobulin synthesis (by isotope incorporation and radio-immunoelectrophoresis) and localization (by fluorescence microscopy).

2. Salivary gland scintigraphy with technetium to evaluate xerostomia.

3. Fluorescence microscopy to detect anti-salivary gland antibodies in serum of patients.

Major Findings:

1. In about 75% of patients with Sjogren's syndrome, the labial salivary glands are heavily infiltrated with lymphoid and plasma cells. These synthesize excessive quantities of IgG, IgM and IgA. Immunofluorescent studies confirm the excessive production of IgM. Control patients (rheumatoid arthritis, systemic lupus erythematosus) do not show lymphoid infiltrates or immunoglobulin synthesis of this degree. A monoclonal IgM was found in three patients with associated Waldenstrom's macroglobulinemia. Another patient with diffuse hypergammaglobulinemia and no evidence of lymphoma was also producing a monoclonal IgM in the lip tissue. The local production of rheumatoid factor by lip tissue from patients with Sjogren's syndrome has been demonstrated.

2. Salivary scintigraphy with technetium is a useful technique for evaluating the extent of salivary gland destruction. Using this method, salivary dysfunction in Sjogren's syndrome can be assigned to one of four grades depending upon the extent of dysfunction. Several patients treated with cyclophosphamide have shown improvement in salivary scintigraphy.

3. Antibody to salivary gland ducts can be demonstrated by immunofluorescence with an antiserum to human immunoglobulins.

4. Sensitization of peripheral blood lymphocytes to salivary gland extracts cannot be demonstrated using an assay system measuring incorporation of H^3 thymidine into DNA.

5. Several new patients with Sjogren's syndrome and lymphoma have been treated. These cases are being reported in a review article currently in preparation.

Significance to Bio-Medical Research and the Program of the Institute:

Sjogren's syndrome, in its relationship to autoimmune disorders, vasculitis and malignant lymphoma, is at the crossroads of the autoimmune and neoplastic lymphoid disorders. The diagnostic value of the labial biopsy has been established. This procedure offers an easily accessible source of immunologically reactive lymphoid cells. The immunobiology of these cells has been studied.

Proposed Course:

A double-blind treatment protocol employing cyclophosphamide or placebo will be initiated at the Clinical Center and in Dr. Talal's new laboratory at the University of California Medical Center. We will attempt to modify the natural progression of the sicca syndrome by a six month course of cyclophosphamide.

Honors and Awards: None

Publications:

1. Steinberg, A. D., and Talal, N.: The coexistence of Sjogren's syndrome and systemic lupus erythematosus. Ann. Intern. Med. 74:55-61, 1971.

2. Steinberg, A. D., Green, W. T., and Talal, N.: Thrombotic thrombocytopenic purpura complicating Sjogren's syndrome. J.A.M.A. 215: 757-761, 1971.

3. Talal, N.: Sjogren's syndrome. In Conn, H. F. and Conn, R. B., Jr. (Eds.): Current Diagnosis 2. Philadelphia, W. B. Saunders Co., 1968, pp. 941-944.

4. Waldmann, T. A., Johnson, J. S., and Talal, N.: Hypogammaglobulinemia associated with accelerated catabolism of IgG secondary to its interaction with an autoreactive monoclonal IgM. J. Clin. Invest. (In press)

5. Schall, G. L., Anderson, L. G., Wolf, R. O., Herdt, J. R., Tarpley, T. M., Cummings, N. A., Zeigler, L. S., and Talal, N.: Sequential salivary scintigraphy in the evaluation of xerostomia in Sjogren's syndrome. J.A.M.A. (in press).

Serial No. NIAMD-ARB-6c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Cyclophosphamide Therapy of Systemic Lupus Erythematosus Renal Disease

Previous Serial Number: 6c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Norman Talal, Dr. H. Benfer Kaltreider (Univ. of California at San Francisco), Dr. Parker J. Staples (Strong Memorial Hospital), Dr. Alfred D. Steinberg, and Dr. Edward J. Goetzl (Peter B. Brigham Hospital).

Cooperating Units: University of California at San Francisco, Strong Memorial Hospital, Rochester, New York and Peter B. Brigham Hospital, Boston, Massachusetts.

Man Years:

Total:	.8
Professional:	.5
Other:	.3

Project Description:

Objectives:

To evaluate the efficacy and side effects of cyclophosphamide therapy in a controlled study of the treatment of systemic lupus erythematosus (SLE) on a short term basis. To obtain information bearing on the relationship of various laboratory parameters to disease activity in SLE.

Methods Employed:

Patients with proven SLE and renal disease are admitted to the Clinical Center. There is an initial two week Control Period during which time baseline and diagnostic clinical and laboratory studies are obtained.

When the above is accomplished, a Treatment Period of 10 weeks begins with the administration of either drug (cyclophosphamide) or placebo. The medication is given in a modified double blind fashion: 1) The "observing" physician and the patient are unaware of either the nature of the medication or the patient's hematologic status; 2) The "therapist" regulates the drug dose according to the absolute granulocyte level.

Throughout the Control and Treatment Periods, the steroid dose is held constant at or below 30 mg. prednisone daily and serial laboratory and clinical observations are made. At the end of the study, the drug code is "broken" and the patient's course evaluated.

Major Findings:

A total of 13 patients, all female, have completed the study. Six received placebo, and 7 cyclophosphamide. Two patients initially treated with placebo were subsequently treated with cyclophosphamide by the same protocol and are included in both cytoxan and placebo groups. Two cyclophosphamide patients did not complete the study. One died of pulmonary embolism in week 5. Another on corticosteroids and salicylates sustained a gastrointestinal hemorrhage in week 6. Other complications in cyclophosphamide treated patients included alopecia in all, hemorrhagic cystitis in one (week 8), and a pseudomonas axillary abscess in one (week 6). One placebo patient was removed from the study because of rapid deterioration.

All cyclophosphamide patients developed lymphopenia and most had reductions of immunoglobulins G and M. Results of the 15 courses of therapy are shown in the table below expressed as net improvement:

	Cyclophosphamide	Placebo
Increased creatinine clearance	2/9	1/6
Improved urinary sediment	3/9	-1/6
Reduced 24 hour urine protein	5/9	-3/6
Rise in Serum complement (C'3)	8/9	-1/6
Fall in anti-DNA antibodies	7/9	1/6
Improved extra-renal disease	5/9	-4/6

The best responses were seen in the patients with the mildest renal disease. Two of the mildest received both placebo and cyclophosphamide courses: of the 6 listed parameters, one patient showed 1 better on placebo and 6 better on cyclophosphamide; the other had 0 better on placebo and 5 better on cyclophosphamide.

It is concluded that cyclophosphamide, although toxic, can bring about an improvement in some patients with lupus nephritis. Means for increasing the efficacy and reducing the toxicity of the drug should be sought.

Significance to Bio-Medical Research and the Program of the Institute:

This study provides long needed controlled data on the efficacy of cyclophosphamide in SLE nephritis on a short term basis. Additional long term studies would be required to decide such matters as effect on survival.

Proposed Course:

Another immunosuppressive agent, azathioprine has been used by other investigators in the therapy of SLE nephritis. The relative efficacy of azathioprine as compared with cyclophosphamide is unknown. We have expanded this study to provide for a comparison between azathioprine, cyclophosphamide, and placebo in SLE nephritis in a manner similar to the present study. A manuscript of the original study is in preparation.

Honors and Awards: None

Publications:

1. Steinberg, A. D., and Decker, J. L.: The Management of Lupus Nephritis. Ann. Intern. Med. 73:1035-1036.

Serial No. NIAMD-ARB-7c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Controlled study of cyclophosphamide and azathioprine in systemic lupus erythematosus with nephritis

Previous Serial Number: NIAMD-ARB-7C

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Alfred Steinberg, Dr. Nortin M. Hadler, and Dr. Kent Salisbury

Cooperating Units: None

Man Years:

Total:	1.8
Professional:	1.3
Other:	.5

Project Description:

Objectives:

An earlier study (Serial No. ARB-6c) has demonstrated that cyclophosphamide has a substantial effect on systemic lupus erythematosus (SLE). Reductions of anti-DNA antibodies and increases in serum complement values were more impressive than was amelioration of renal disease. Azathioprine is being widely used in the same situations although no well controlled detailed trials are yet reported. Others have shown that cyclophosphamide has substantially more effect on NZB/BW mouse renal disease, a model of lupus nephritis, than does azathioprine.

In view of the significant toxicity of both drugs, it is important to compare them directly within a single trial. The objectives are to assess the therapeutic efficacy/toxicity of azathioprine and cyclophosphamide in comparison to each other and to placebo in SLE nephritis.

Methods Employed:

About 40 patients meeting predefined criteria for diagnosis and activity of nephritis will be admitted to the 12 week trial. In the two week baseline control period, the patients will be kept on the anti-inflammatory regimen (usually prednisone and aspirin) needed to control extra-renal manifestations.

These will be continued into the ten week study period with as little change as possible and with the addition of randomly assigned cyclophosphamide, azathioprine or placebo. Dosage of each drug will be 3-4 mg/kg/day unless leukopenia (less than 3,000 WBC/mm^3) requires reduction below that level.

A large number of serological, chemical, and urine changes will be followed in the course. Immunoglobulin levels, anti-DNA antibodies, serum complement (by hemolytic and by precipitin assay), urinary fibrin split products, and the response to a number of antigens (for delayed hypersensitivity and circulating antibodies) are among those parameters.

Major Findings: None.

Significance to Bio-Medical Research and the Program of the Institute:

If differences in the response of the disease and some of the immunological processes can be detected between the alkylating agent and the purine analogue, it will be possible to draw conclusions in reference to the cell populations involved in systemic lupus erythematosus and thus extend our understanding of the disease process itself. It is expected that the results will permit us to select between the two in ordinary clinical management.

Proposed Course:

The study will be carried through as outlined over the next two or three years.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-8c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Systemic Lupus Erythematosus Coagulation Study

Previous Serial Number: NIAMD-ARB-8c

Principla Investigator: Dr. John L. Decker

Other Investigators: Dr. Alfred D. Steinberg, Dr. Harvey Gralnick (CC-CP) and Dr. Sally Marchesi (CC-CH)

Cooperating Units: Clinical Center, Clinical Pathology Department

Man Years:

Total:	.5
Professional:	.3
Other:	.3

Project Description:

Objectives:

To study abnormalities of coagulation in patients with systemic lupus erythematosus (SLE), and to correlate these abnormalities with disease activity.

Methods Employed:

Patients with SLE who are admitted to the clinical center and selected patients with SLE seen in the out-patient department are studied.

Evaluation of clinical status is done independently by Drs. Decker and Steinberg and the results recorded on separate evaluation sheets. On the same day, the patient's blood is studied for coagulation factors, fibrin split products, and other hematological parameters. A 24-hour urine is also studied for fibrin split products. The coagulation study by clinical hematology is performed without knowledge of the clinical evaluation.

Major Findings: None

Significance to Bio-Medical Research and the Program of the Institute:

This study complements nicely our other interests in SLE. It is hoped that the study may provide another index of disease activity independent of our usual immunological parameters. Perhaps the extent of fibrin split products will correlate with disease activity in such a way that therapy can be based on their presence. Finally, the study may provide further insights into the pathophysiology of SLE.

Proposed Course:

A two year study of 20 to 30 patients and serial evaluation over that period of time.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-9c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Infection in Systemic Lupus Erythematosus

Previous Serial Number: NIAMD-ARB-5C

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Parker J. Staples (Strong Memorial Hospital), Dr. Robert S. Gordon (A-CI), and Dr. Dale Gerding (CR-OD)

Cooperating Units: Strong Memorial Hospital, Rochester, New York; NIAMD, Clinical Investigations; Division of Computer Research and Technology, Office of the Director

Man Years:

Total:	.5
Professional:	.3
Other:	.3

Project Description:

Objectives:

To clarify the incidence and prevalence of infection in patients with systemic lupus erythematosus (SLE), with special emphasis on identifiable predisposing factors.

Methods Employed:

The work is based on the Clinical Center records of patients with known SLE. Control groups of patients with either definite rheumatoid arthritis (RA) or nephrotic syndrome (NPS) are also being studied.

Information on patients has been obtained regarding estimates of clinical disease activity, drug types and doses, renal status, factors known to be associated with infection, e.g., diabetes mellitus, selected serial laboratory data, and number and type of infections observed. All data has been coded and placed on magnetic tape. Computer programs have been written which analyze the data for number and type of infection, antecedent drug history, clinical disease activity, renal status and the like.

Major Findings:

Twenty-three patients with SLE, 20 with RA, and 11 with NPS were analyzed during 5,294 days of hospitalization. Thirty nine episodes of bacterial or mycotic infection were identified. The infection rate (IR - episodes of infection per 100 days of observation) was 1.64 for SLE and 0.16 for non-SLE (0.0 for RA and 0.23 for NPS).

IR in both SLE and NPS was found to increase in proportion to the dose of adrenal corticosteroid administered. At all dose levels in IR in SLE exceeded IR in NPS.

The IR also increased as clinical disease activity increased in both SLE and NPS. There was no clear relationship of IR to various parameters of renal disease except that azotemia (BUN >60mg%) was associated with an IR of 3.2 in SLE and 0.0 in NPS.

Significance to Bio-Medical Research and the Program of the Institute:

It has always been held that infections are more common in SLE than can be accounted for by potential renal failure and/or adrenal corticosteroid therapy. This study provides good evidence to support this notion. Machien data handling capability has rarely been applied to this kind of work and, in itself, constitutes a departure of significance.

Proposed Course:

Analysis of the data continues. Unfortunately the data base has proven too small to provide substantially more information than is mentioned above under Major Findings.

The study has demonstrated the merits of recording patient medication intake directly in machinable form and efforts are underway to make this automatic in all our patients.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-10c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Leukopenia in Connective Tissue Disease

Previous Serial Number: NIAMD-ARB-15c

Principal Investigator: Dr. Norman Talal

Other Investigators: Dr. John L. Decker, Dr. Sheldon Wolff (I-LCI) and Dr. Harry Kimball (I-LCI)

Cooperating Units: National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigation

Proposed Course:

The project has been set aside for the moment because of lack of specific type of patients.

Serial No. NIAMD-ARB-11c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Antibodies to Double Stranded DNA and RNA in Spontaneous and Drug-Induced Systemic Lupus Erythematosus

Previous Serial Number: NIAMD-ARB-13C

Principal Investigator: Dr. Norman Talal

Other Investigators: Dr. Alfred Steinberg, Dr. Peter H. Schur (Robert Breck Brigham Hospital)

Cooperating Units: Robert Breck Brigham Hospital, Boston, Massachusetts

Man Years:

Total:	1.0
Professional:	.5
Other:	.5

Project Description:

Objectives:

Study the specificity of anti-DNA and anti-RNA antibodies in human and murine lupus and their frequency in other diseases.

Methods Employed:

Farr ammonium sulfate precipitation assay, using C^{14}-KB cell DNA or C^{14} polyinosinic·polycytidylic acid as antigen.

Major Findings:

1. Antibodies to double stranded DNA and RNA are found respectively in 75% and 45% of human lupus patients, and rarely in other human autoimmune disorders, including drug-induced lupus.

2. Inhibition studies demonstrate that these are largely distinct antibody populations that may be directed against viral nucleic acids.

Antibodies to RNA have specificity for several double-stranded RNAs investigated. Such antibodies arising spontaneously in B/W mice seem to have greater affinity for RNA than similar antibodies appearing in human lupus. Reovirus RNA is the most efficient inhibitor of C^{14} poly I·poly C binding.

Significance to Bio-Medical Research and the Program of the Institute:

The presence of antibodies to double-stranded DNA and RNA strengthens the possibility of viral antigens in human lupus.

Proposed Course:

Continued clinical study of anti-DNA and anti-RNA antibodies in SLE with emphasis on immunologic specificity, employing natural and synthetic nucleic acids as specific inhibitors.

Honors and Awards: None

Publications:

1. Talal, N.: Immunologic and viral factors in the pathogenesis of systemic lupus erythematosus. Arthritis Rheum. 13:887-894, 1970.

2. Schur, P. H., Stollar, B. D., Steinberg, A. D., and Talal, N.: Incidence of antibodies to double-stranded RNA in systemic lupus erythematosus and related diseases. Arthritis Rheum. (In press).

3. Talal, N., Steinberg, A. D., and Daley, G.: Inhibition of antibodies binding polyinosinic·polycytidylic acid in hum an and mouse lupus sera by viral and synthetic ribonucleic acids. J. Clin. Invest. (In press).

Serial No. NIAMD-ARB-12c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Pathogenesis of Auto-Immunity in NZB and NZB/NZW F_1 Mice

Previous Serial Number: NIAMD-ARB-12C

Principal Investigator: Dr. Norman Talal

Other Investigators: Dr. Thomas Chused, Dr. Michael Jacobs, Dr. Alfred D. Steinberg, Dr. Adi Gazdar (C-VLL), Dr. Deborah Carpenter (BS-LP), Dr. Peter Schur (Robert Breck Brigham Hospital).

Cooperating Units: National Cancer Institute, Viral Leukemia and Lymphoma Branch; Division of Biologic Standards, Laboratory of Pathology; Robert Breck Brigham Hospital, Boston, Massachusetts.

Man Years:

Total:	2.8
Professional:	2.3
Other:	.5

Project Description:

Objectives:

The NZB and B/W mice are genetically predisposed to develop Coomb's positive hemolytic anemia, lupus glomerulonephritis, positive LE cells, anti-nuclear factor, and anti-DNA antibodies. In later life, NZB mice also develop malignant lymphomas. These mice represent an excellent laboratory model for studying the pathogenesis of autoimmune disease.

Methods Employed:

1. Assay for anti-DNA and anti-RNA antibodies by $(NH_4)_2SO_4$ binding assay employing C^{14}-DNA prepared from KB cells or C^{14} polyinosinic·polycytidylic (poly I·poly C) acid.

2. Induction of tolerance to sheep erythrocytes with cyclophosphamide; transfer of spleen, bone marrow or thymus cells into lethally irradiated syngeneic hosts to evaluate functional properties of these cooperating cell populations, using Jerne plaque assay.

3. Adoptive transfer of spleen, thymus and bone marrow cells from anti-DNA positive donor mice into x-irradiated anti-DNA negative syngeneic recipients to study the tissue origin of the cells making this abnormal antibody.

Major Findings:

1. Cyclophosphamide-induced tolerance to sheep erythrocytes can be transferred when bone marrow and thymus cells are injected together into syngeneic x-irradiated recipients. When either population from C57Bl mice is injected with the reciprocal normal (i.e. non-tolerant) population, tolerance is again transferred. Thus, both bone marrow and thymus of C57Bl mice can transfer the donor animal's tolerant status. Likewise, bone marrow from B/W mice will also transfer tolerance when injected with normal B/W thymus. However, B/W thymus from tolerant donors fails to hasten a state of tolerance, indicating an abnormality of the B/W thymus.

2. B/W spleen from anti-DNA positive donors will transfer "auto-antibody" production into irradiated syngeneic recipients. B/W thymus and bone marrow alone or combined are unable to so. Likewise, anti-DNA antibody is not produced when spleen cells from anti-DNA negative donors are transferred. Thus, cells producing this abnormal antibody are located in the B/W spleen.

3. B/W mice may be under the influence of a natural adjuvant and are relatively resistant to the adjuvant properties of poly I·poly C.

4. Moloney leukemia virus accelerates anti-RNA antibodies in B/W mice. This acceleration is prevented if the mice are first made tolerant to RNA by treatment with poly I·poly C and cyclophosphamide.

Significance to Bio-Medical Research and the Program of the Institute:

Our studies have led to a hypothesis concerning the pathogenesis of human and murine lupus. According to this concept, genetic, immunologic and viral factors contribute to the disorder. Because of a genetic lesion, an immunologic imbalance arises in which antibody formation is augmented and cellular immunity is depressed. This condition leads to a breakdown of tolerance to viral and other antigens, and to the formation of anti-nucleic acid (? anti-viral) antibodies. Probably, different common viruses can trigger this event.

Proposed Course:

1. Attempt to validate this hypothesis through studies of virus infected control and tolerant mice.

2. Attempt to utilize tolerance regimen (poly I·poly C and cyclophosphamide) to treat human and murine lupus.

Honors and Awards: None

Publications:

1. Steinberg, A. D. and Talal, N.: Suppression of antibodies to nucleic acids with polyinosinic polycytidylic acid and cyclophosphamide in murine lupus. Clin exp. Immunol. 7:687-691, 1970.

2. Carpenter, D. F., Steinberg, A. D., Schur, P. H., and Talal, N.: The pathogenesis of autoimmunity in New Zealand Mice. II. Acceleration of glomerulonephritis by polyinosinic·polycytidylic acid. Lab. Invest. 23:628-634, 1970.

3. Steinberg, A. D., Pincus, T., and Talal, N.: The pathogenesis of autoimmunity in New Zealand mice. III. Factors influencing the formation of anti-nucleic acid antibodies. Immunology (In press).

4. Talal, N. and Steinberg, A. D.: Immunity and tolerance to synthetic polynucleotides in New Zealand mice. In Beers, R. F. and Braun, W. (Eds.): Biological Effects of Polynucleotides. New York, N. Y., Springer-Verlag, p 336-351 (In press).

5. Steinberg, A. D., Chused, T. M., Jacobs, M. E., and Talal, N.: Cyclophosphamide tolerance as a test for antigenicity of nucleic acids. Nature 228:1090, 1970.

6. Steinberg, A. D., Law, L. D., and Talal, N.: The role of the NZB/NZW F_1 thymus in experimental tolerance and autoimmunity. Arthritis Rheum 13:369-377, 1970.

7. Steinberg, A. D., Baron, S., Uhlendorf, C., and Talal, N.: Depression of the interferon response to polyinosinic·polycytidylic acid by specific antibody. Proc. Soc. Exp. Biol. Med. (In press).

8. Gazdar, A. F., Beitzel, W., and Talal, N.: The age-related response of New Zealand mice to a murine sarcoma virus. Clin. Exp. Immun. (In press).

9. Talal, N., Steinberg, A. D., Jacobs, M. E., Chused, T. M., and Gazdar, A. F.: Immune cell cooperation, viruses and antibodies to nucleic acids in New Zealand mice. J. Exp. Med. (In press).

Serial No. NIAMD-ARB-13c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on a Mouse Myeloma Protein Having Antibody Activity

Previous Serial Number: NIAMD-ARB-18c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Edward J. Goetzl and Dr. Nortin M. Hadler

Cooperating Units: None

Man Years:

Total:	1.3
Professional:	.8
Other:	.5

Project Description:

Objectives:

As outlined previously the availability of homogeneous immunoglobulins with specific binding activity chemically indistinguishable from conventional antibody activity makes it possible to conduct studies which had been essentially impossible to perform on heterogeneous antibodies: structural details of combining sites, role of light and heavy chains in the formation of the combining sites, etc.

Methods Employed:

It was previously shown that the nitrophenyl binding γA myeloma protein from the mouse tumor MOPC 315 could be 'affinity labeled' with meta-nitrobenzene diazonium fluoroborate. A single tyrosine - Tyr34 - on the light chain was thereby derivatized. We have attempted to reduce the azo-tyrosine bond in situ with dithionide with the hope that 3 aminotyrosine (3AT) would thereby be generated. Since the aryl amino group of 3AT has an unusually low pK, it should be possible to selectively derivatize it with cross-linking reagents.

Major Findings:

1. Affinity labeled protein 315 could be reduced with dithionite such that 3 aminotyrosine was generated and considerable activity recovered.

2. Under conditions where no reaction occurred with the native protein difluoro-dinitro benzene (F_2DNB) and p,p' difluoro-m,m' dinitrobenzene diphenyl sulfone (F_2DPS) reacted with the affinity labeled reduced protein.

3. With F_2DNB but not with F_2DPS evidence was obtained that cross-linking had occurred; that is the second fluoride group was no longer capable of being hydrolyzed off (in 1N NaOH) to give a spectrally detectable nitrophenolate ion.

4. Evidence was obtained that with F_2DNB two types of cross-links had occurred: between Tyr_{34} on the light chain and another residue on the same light chain and, alternatively, between Tyr_{34} on the light chain and a residue in the Fd region of the heavy chain.

Significance to Bio-Medical Research and the Program of the Institute:

Our own work and that of others has already shown that much useful data on the structure of antibody combining sites can be obtained by the use of homogeneous mouse myeloma proteins with recognizable binding activity. Affinity labeling studies on both myeloma proteins and conventional antibodies have shown that when diazonium reagents are used, azotyrosine is a frequent product. The techniques developed in the present study should have wide application.

Proposed Course:

1) The cross-linked peptides will be isolated and sequenced, thereby giving information on what regions of the heavy and light chains are close to Tyr_{34} in the combining site. 2) Other cross-linking reagents will be tried with protein 315. 3) Other affinity labeled proteins will be studied using F_2DNB and F_2DPS.

Honors and Awards: None

Publications:

1. Goetzl, E. J. and Metzger, H.: Affinity labeling of a mouse myeloma protein which binds nitrophenyl ligands. Sequence and position of a labeled tryptic peptide. Biochemistry 9:3862-3871, 1970.

2. Hadler, N. M. and Metzger, H.: Site directed cross-linking: A new approach to mappling antibody combining sites. Proc. Natl. Acad. Sci., 1971. (In press).

Serial No. NIAMD-ARB-14c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July, 1970 through June 30, 1971

Project Title: Use of Tetranitromethane to Study Antibody Combining Sites

Previous Serial Number: None

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Neil Otchin
Mr. John Lee

Cooperating Units: None

Man Years:

Total:	1.3
Professional:	.8
Other:	.5

Project Description:

Objectives:

These studies are intended to shed further light on the structure of immunoglobulin combining sites. Tetranitromethane will more or less specifically introduce nitro groups into the 3 position of tyrosyl groups in proteins under mild conditions. Since tyrosyl groups appear to play an important role in many antibody combining sites we wished to see if modification of such tyrosines would effect the combining activity.

Methods Employed:

Tetranitromethane is used to modify myeloma proteins with defineable binding activity. The amount of nitro-tyrosine which is incorporated is assessed by taking advantage of the spectral properties of nitro-tyrosine. Nitration is carried out in the presence and absence of ligand to see a) if the presence of the ligand effects the level of nitration observed and b) to see if the effects of nitration can be modified. Changes in the activity of the proteins are assessed by equilibrium dialysis.

Major Findings:

1) Studies with γM_{Wag} - γM_{Wag} is a human Waldenstrom macroglobulin which binds nitrophenyl ligands. We have found that at very low levels of nitration (~1 mole nitrotyrosine/mole combining sites there is almost complete loss of binding activity. In the presence of a 'protector' N(4 nitrophenyl) NH_2 caproate the rate of nitration is retarded and at comparable levels of nitration considerably less activity is lost.

2) Studies with protein from TEPC 15 - The γA myeloma protein from the mouse plasmacytoma TEPC 15 binds phosphoryl choline. We have found that at very low levels of nitration 1-2 moles nitrotyrosine per mole combining sites two changes in the binding activity of the protein are observed. a) there is a decrease in the association constant to about 1/2 the value observed with the native protein. This effect of nitration is not influenced by the presence of "protecting" ligand b) there is a loss in the number of combining sites with a detectable binding constant. This effect can be protected against by the presence of the ligand phosphoryl choline.

Significance to Bio-Medical Research and the Program of the Institute:

Nitration of tyrosines is a particularly useful test for probing combining sites for the following reason. The nitrotyrosine can be readily reduced to 3-aminotyrosine (3AT) with dithionite. By virtue of its low pK the aryl amino group of 3AT can be selectively modified with such reagents as radioactive dinitrofluorobenzene, dansylchloride, cross-linking reagents etc. This will provide an opportunity to relatively easily identify the peptides containing the tyrosines in question and to conduct "mapping" experiments with the cross-linking reagents. These studies are part of a series of studies being conducted in this laboratory intended to further our understanding of the structure of immunoglobulin combining sites.

Proposed Course:

After some further experiments to substantiate the preliminary results detected above we will try to identify which polypeptide chains and which tyrosines on those chains are the ones responsible for the effects seen.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-15c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunoglobulins on Cell Surfaces

Previous Serial Number: 20C

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Albert Sullivan, and Dr. Philip M. Grimley

Cooperating Units: Section on Pathological Anatomy, NCI

Man Years:

Total:	1.5
Professional:	1.0
Other:	.5

Project Description:

Objectives:

Essentially all the important activities of antibodies are ultimately expressed not in free solution but on cell surfaces. For example antibodies act as antigen receptors on cells destined to respond in the immune response. Similarly it is γE on effector cells which when stimulated by allergens, leads to histamine release. Specific antigen catabolism similarly must be mediated by antigen antibody complexes interacting with phagocytic cells. We are now establishing a variety of experimental situations whereby we can look at such systems.

Methods Employed:

Our initial efforts will be directed to making highly specific anti-immunoglobulin. By employing ferritin labeled antibody reagents and electronmicroscopy we hope to gain a better understanding of the distribution of such immunoglobulin receptor molecules.

Major Findings:

Our main attention has so far been directed towards defining the cellular receptors for γE immunoglobulin. Anti-human γE was produced in burros and

the antibodies purified on columns of human γE coupled to Sepharose. Rabbit antibodies induced against horse ferritin and burro γG were purified in an analogous manner. Peptic $F(ab')_2$ fragments prepared from each of the purified rabbit antibodies were reduced, mixed and reoxidized. Hybrid $F(ab')_2$ fragments (containing 1 site directed against ferritin and 1 site directed against burro γG were specifically purified.

Human peripheral leukocytes were enriched for basophils by a gradient technique. Cells were incubated without added immunoglobulin, with added human γE or γG. After washing they were incubated with normal burro γG, burro-anti γE or γG. After thorough washing they were incubated with the rabbit $F(ab')_2$ hybrid antibodies. The cells were sectioned and examined by electronmicroscopy. The following conclusions can so far be drawn:

1. γE can be found only on basophils.

2. The amount of γE on the basophils increases dramatically if the cells are pre-incubated with human γE.

3. In either case the γE is distributed in patches.

4. Excellent micrographs can be obtained which define the location of the immunoglobulin on the cell. Control studies indicate that non-specific sticking of immunoglobulins is not a problem.

Significance to Bio-Medical Research and the Program of the Institute:

It is of considerable importance to study the behavior of antibodies on the surface of cells in order to more completely understand the mechanism of the immune response. It is on the cell surface that most of the critical steps of the immune response transpire and it is here that one must direct one search for the ultimate mechanisms by which antigen-antibody reactions initiate specific cellular responses.

Proposed Course:

Further studies will be done to see if the basophils of "responder" and "non-responder" individuals can be distinguished.

In addition receptors for γG and γG and γM receptors on the surfaces of lymphocytes (both normal and leukemic) will be examined for using the same techniques.

Honors and Awards: None

Publications:

Metzger, H.: The antigen receptor problem. Ann. Rev. Biochem. 39:889-929 (1970).

Serial No. NIAMD-ARB-16c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Combining Sites of Mouse Myeloma Proteins Which Bind Phosphoryl Choline

Previous Serial Number: None

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Bruce Chesebro

Cooperating Units: None

Man Years:

Total:	1.5
Professional:	1.0
Other:	.5

Project Description:

Objectives:

One of the most common activities found among mouse myeloma proteins is the ability to bind phosphoryl choline or its analogues. Since there already exist 6-8 such proteins which share this property but which are clearly distinguishable on the basis of their binding constant and specificity, these proteins therefore seem ideally suited to study structural functional relationships in immunoglobulin combining sites. Furthermore, since the ligand phosphoryl choline is quite different than the aromatic ligands which have been most frequently studied in immunochemistry some new features of the combining sites of these proteins may become evident.

Methods Employed:

To begin our work it was necessary to purify the proteins and to obtain a suitable labeling reagent. We were able to accomplish both by synthesizing the compound pNO_2 phenyl phosphoryl choline (PNPC) a compound for which no reproducible synthesis had heretofore existed.

Major Findings:

1. PNPC was synthesized utilizing methyl iodide, pNO_2 phenylphospho-dichloridate and dimethylaminoethanol. In this way it was possible to make the radioactive compound by using radioactive methyl iodide. The PNPC could be reduced to give the corresponding aniline analogue and the latter could be diazotized to give p-azophenylphosphoryl choline.(PAPC)

2. An effective immune adsorbent could be made by diazotizing PAPC to glycyl tyrosyl Sepharose. This adsorbent yields highly purified preparations of 4 different phosphoryl choline binding myeloma proteins.

3. PAPC has proven to be an effective affinity labeling reagent for these proteins. In all cases there is a much more rapid rate of reaction with the appropriate myeloma protein them with a non-specific protein and the reaction can be effectively blocked by the presence of phosphoryl choline in the incubation mixture.

4. The site of labeling has been examined for in four proteins. In two instances the major amount of label goes on the light chain, in the other two on the heavy chains. In the two cases where light chains are being labeled exclusively 'all' of the label is on a tyrosine. The labeled heavy chain residue has not yet been determined.

5. The labeled light chain tyrosyl peptide from one of the proteins has been isolated and is now being sequenced.

Significance to Bio-Medical Research and the Program of the Institute:

The significance of studying functional myeloma proteins has been discussed in previous Progress Reports. As indicated in the introduction the particular set of proteins should be unusually instructive as far as studying immunoglobulin combining sites.

Proposed Course:

Labeled peptides will be isolated from each of the affinity labeled proteins and their sequences determined - other proteins which have similar activities will be sought. In this way, it is hoped that meaningful patterns will become discernible.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-17c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanism of Immunoglobulin Chain Recognition

Previous Serial Number: 21C

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Neil Otchin

Cooperating Units: None

Man Years:

Total:	1.3
Professional:	.8
Other:	.5

Project Description:

Objectives:

On recombining the separated chains of myeloma proteins it has been found that autologous chains tend to recombine preferentially. This suggests a non-random mechanism by which light and heavy chain pairs are chosen by a cell for synthesis of specific immunoglobulins. It is also possible that the experimental findings result from a failure to fully unfold the respective chains so that the remaining structure influences the chain recombination. We intend to study this phenomenon by fully unfolding the immunoglobulin chains to see if they will then continue to demonstrate preferential recombination.

Methods Employed:

The polypeptide chains of several myeloma proteins are being individually labeled. A protein will be chosen in which the preferential recombination phenomenon can be clearly observed. Such chains will then be completely unfolded in guanidine and then refolded. Such refolded chains will then be restudied for preferential chain recombination.

Major Findings:

Because of major difficulties encountered in attempts to reassemble immunoglobulin molecules from chains which have been completely reduced and unfolded and because of theoretic considerations this project has been discontinued.

Proposed Course:

See Major Findings.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB- 18c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on a Waldenstrom Macroglobulin Which Binds Nitrophenyl Ligands

Previous Serial Number: 19C

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Robert Ashman
Dr. Allan P. Kaplan

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.3
Other:	.5

Project Description:

Objectives:

Preliminary studies on the binding properties of γM_{Wag} - a Waldenström macroglobulin have been completed. We are now investigating this protein to see if we can find any evidence for conformational changes in the protein subsequent to binding of ligand.

Methods Employed:

We studied the protein using circular dichroism, ^{3}H-exchange and susceptibility to proteolytic digestion according to standard methods.

Major Findings:

See previous report (NIAMD-ARB-19C, 1969-1970). We concluded from our studies that the only changes which could be clearly assigned were those occurring in the Fab regions of the molecule. These could have resulted from a) a small change in the conformation of the Fab region b) a small change in thermal segmental motion of the Fab region c) 'shielding' of certain amino-acid side chains or peptide bonds in the region of the combining site by the ligand. We could find no evidence for distant effects

(e.g. in the Fc region of the molecule) when ligand was bound.

Significance to Bio-Medical Research and the Program of the Institute:

Antibodies serve as mediators - in antigen stimulation of the immune response it is antibodies on the surface of immunocompetent cells which are the critical 'triggers'; similarly in cellularly mediated anayphylaxis, rejection phenomenon and antigen catabolism it is cell surface antibodies which give the requisite specificity. Presumably some of these reactions involve changes in antibody conformation subsequent to antigen binding. We are investigating the usefulness of the homogeneous Waldenström macroglobulins for studying such phenomena. Clearly this is an area which has considerable importance in obtaining a further understanding of the molecular mechanisms involved in the immune response.

Proposed Course:

This study is complete.

Honors and Awards: None

Publications:

Ashman, R. F., Kaplan, A. P. and Metzger, H.: A search for conformational change on ligand binding in a human γM macroglobulin. I. Circular dichroism and tritium exchange. Immunochemistry 8, 1971 (in press).

Ashman, R. F. and Metzger, H.: A search for conformational change on ligand binding in a huamn γM macroglobulin. II. Susceptibility to proteolysis. Immunochemistry 8, 1971 (in press).

Serial No. NIAMD-ARB-19c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Structural Analysis of a Human Macroglobulin

Previous Serial Number: 17C

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Allan Kaplan (Robert Breck Brigham Hospital), Dr. Leroy Hood (C-I) and Dr. William Terry (C-I)

Cooperating Units: Robert Breck Brigham Hospital (Boston, Massachusetts; National Cancer Institute, Immunology Branch

Man Years:

Total:	0
Professional:	0
Other:	0

Proposed Course:

This project led to a publication and is now complete.

Publications:

Kaplan, A. P., Hood, L. E., Terry, W. D. and Metzger, H.: Amino Terminal sequences of human immunoglobulin heavy chains. Immunochemistry, 1970, (in press).

Serial No. NIAMD-ARB-20C
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Enrichment of Antigen-Receptor Bearing Immune Cells

Previous Serial Number: None

Principal Investigator: Dr. Paul H. Plotz

Other Investigators: Dr. Kent Salisbury

Cooperating Units: None

Man Years:

Total:	2.0
Professional:	1.3
Other:	.8

Project Description:

Objectives:

To obtain enriched population of immune cells so that the antigen receptor, site of initial antigen recognition, may be studied in order to understand how antigens initiate immune responses.

Methods Employed:

Lymphoid cells from immunized mice are exposed to polymer beads with antigen coupled to their surface. Cells are studied before and after exposure to beads to determine whether there is depletion of immune compentence. Attempts are made to remove cells sticking to the beads.

Lymphoid cells from immune mice are exposed to antigen in solution and then exposed to macrophage cultures to determine if immune competent cells are specifically removed.

Lymphoid cells from immune mice are serially diluted and passaged in irradiated recepients in order to develop clones of antibody producing cells. Isoelectric focussing in polyacrylamide gels is used to follow antibody heterogeneity. If clones are found they will be used in studies to find the receptor.

Major Findings: None

Significance to Bio-Medical Research and the Program of the Institute:

Understanding of foreign antigen recognition and the triggering process in immunity will advance understanding of the origin of the abnormal immune responses seen in patients with autoimmune diseases.

Proposed Course:

The studies will continue.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-21c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Metabolic Transformations of Certain Compounds that May be Significant in Human Health and Disease

Previous Serial Number: None

Principal Investigator: Dr. Peter Goldman

Other Investigators: Dr. Mark A. Peppercorn
Dr. Sheldon Milstien

Cooperating Units: None

Man Years:

Total:	2.0
Professional:	1.5
Other:	.5

Project Description:

Objectives:

To elucidate the origins and normal fate of compounds appearing in human body fluids whose role in human metabolism has not been previously understood.

Methods Employed:

Germ-free rats have been used to decide if the metabolism of certain compounds are of microbiol or host origin. Micro-organisms isolated from human feces and other bacteria commonly found in the human gastrointestinal tract have been tested for their ability to metabolize drugs and other compounds which may be important to human health. The organisms isolated have been characterized and the metabolic products identified by biochemical techniques.

Major Findings:

The metabolism of caffeic acid, previously thought to be carried out by the intestinal microbiota of man and experimental animals, has been examined in a number of bacteria insolated from human feces. It has been found that no individual organism has the ability to catalyze more than one reaction of the series which converts caffeic acid either to m-hydroxyphenylpropionic

acid or 4-ethylcatechol. It has been found that of the organisms tested only a mixed culture of E. coli and Group D Streptococci is capable of catalyzing the dehydroxylation of 3,4-dihydroxyphenylpropionic acid to yield m-hydroxyphenylpropionic aicd. Another strain of Group D Streptococcus, isolated from the same individual, is not capable of participating in this reaction. A similar difference between strains is found in Clostridium perfringens where only one of two strains is capable of reducing caffeic acid.

Significance to Bio-Medical Research and the Program of the Institute:

In experimental animals it has been found that the character of the intestinal microflora determine many aspects of the well-being of the host. It has been difficult to assess factors of this kind in the human but recent evidence suggests that they may be important. For example, the character of the diet and the metabolic potential of the intestinal microflora seem to correlate with the incidence of cancer of the colon in certain population studies and certain compounds of intestinal origin have been implicated in the pathogenesis of the uremic syndrome. The elucidation of the mechanisms involved in phenomena of these kinds might offer the basis for new approaches to preventive medicine and therapeutics.

Proposed Course:

The role of the intestinal microflora in the metabolism of certain trace compounds of dietary origin will be explored. In addition an attempt will be made to measure and examine the significance of some of the compounds apparently associated with the uremic syndrome.

Publications: None

Honors and Awards: None

Serial N. NIAMD-ARB-22c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: High Resolution Analysis of the Urine of Normal Volunteers and Patients taking a Synthetic Diet.

Previous Serial Number: None

Principal Investigator: Dr. Peter Goldman

Other Investigators: Dr. Donald S. Young (CC:CP)

Cooperating Units: Clinical Center, Clinical Pathology Department

Man Years:

Total:	.5
Professional:	.3
Other	.3

Project Description:

Objectives:

To determine whether it is possible to simplify the number of ultraviolet-absorbing and carbohydrate-reactive components that are found in the urine of subjects on normal diets by using a wholly synthetic diet. To determine which components of the urine reflect human physiology rather than diet and to see whether some of these components are altered in various diseases.

Methods Employed:

Normal volunteers or patients are given alternate periods of standard house diet and the totally synthetic Vivonex diet. Analyses of the electrolytes of blood and urine are made and the blood is also analyzed for the standard clinical components. In addition, the urine is analyzed for carbohydrate, ultraviolet-absorbing and ninhydrin-reactive components (amino acids and others). Comparisons are made of the analyses of body fluids while the patients are on the Clinical Center diet and on the synthetic diet.

Major Findings:

On the synthetic diet all normal volunteers showed a decrease in serum urea nitrogen and glucose but a rise in uric acid. The high resolution analysis of the urinary components indicates that by the fifth day of the

diet a new steady state is reached in which a marked simplification of the number of compounds is seen.

Significance to Bio-Medical Research and the Program of the Institute:

The accurate measurement of compounds in urine and serum that have previously not received attention may offer the opportunity to develop new chemical approaches to the evaluation of human health and disease. Metabolic alterations occurring in certain diseases may be discovered which will lead to a further understanding of the disease process. In addition, by establishing "normal values" for compounds in human body fluids, new diagnostic aids for the early detection of disease may be developed.

Proposed Course:

It is intended to continue the analysis of body fluids in normal volunteers under defined conditions and to begin to study the components in urine and serum under these conditions of patients with various diseases.

Honors and Awards: None

Publications: None

Serial No. NIAMD-ARB-23c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Characteristics of the Carbon-Halogen Bond in Compounds of Biological Importance

Previous Serial Number: 24C

Principal Investigator: Dr. Peter Goldman

Other Investigators: Mr. Jay C. Unkeless

Cooperating Units:

Man Years:

Total:	1.0
Professional:	.5
Other:	.5

Project Description:

Objectives:

To elucidate the enzyme mechanisms involved in the cleavage of the carbon-halogen bond and to study the usefulness of compounds containing the carbon-fluorine bond in determining the chemical basis of biological phenomena.

Methods Employed:

γ-Fluoroglutamate has been synthesized by organic chemical methods and the diastereomers separated by column chromatography. Specific enzymes were used to resolve the racemates of the diastereomers. The resolved isomers of γ-fluoroglutamate have been tested as substrates or inhibitors of enzymes or enzyme systems normally reactive with glutamate; A kinetic analysis of the analogue in comparison with glutamate has been made for a number of enzymes.

Major Findings:

Racemates of the erythro and threo diastereomers of γ-fluoroglutamate (FGlu) have been separated by chromatography on Dowex 1; and the enantiomers of each diastereomer have been resolved by specific enzymes. All enzymes

tested which are specific for one enantiomer of glutamate (Glu) reacted also with an enantiomer of FGlu. These enzymes made it possible to relate the configuration of the α-carbon of FGlu to that of Glu. The following enzymes are consistent in relating the configurations of Glu and FGlu in this manner: Glutamate decarboxylase of E. coli (E.C.4.1.1.15), carboxypeptidase G (E.C.3.4.2.6), glutamine synthetase of E. coli (E.C.6.3.1.2), D-glutamate cyclase (E.C.4.2.1.54), glutamate dehydrogenase (E.C.6.3.1.2) and leucine aminopeptidase (E.C.3.4.1.1). Leucine aminopeptidase had been found to be specific for L-leucyl-L-FGlu in a mixture of L-leucyl-DL-FGlu; hence each of the racemates separated by Dowex 1 chromatography was derivatized with L-leucine and treated with leucine aminopeptidase to obtain either pure L-erythro-FGlu or L-threo-FGlu from their respective racemates.

To establish the relative configuration of 2 asymmetrical carbon atoms in FGlu, each diastereomer was related to one of the diastereomers of 4-fluoroproline. A racemate of FGlu was pyrolized to its corresponding 3-fluoro-2-pyrrolidone-5-carboxylic acid which was reduced to its prolyl alcohol. As its benzoyl derivatiev this was related by gas-liquid chromatography to the prolyl alcohol obtained by reduction of either cis or trans 4-fluoroproline of known relative configuration.

D-glutamate cyclase, an enzyme which catalyzes the reaction

$$\text{D-Glu} \rightleftarrows \text{2-pyrrolidone-5-carboxylic acid} + H_2O$$

reacts also with both diastereomers of FGlu to form the corresponding lactams. The presence of the fluorine substituent in both diastereomers accelerates the V_{max} for enzymatic lactam hydrolysis 56 fold in comparison with the natural substrate. Thermodynamic studies with this enzyme indicate that lactam formation from Glu is accompanied by a H of 2.3 kcal, while H for cis-FGlu is 3.9 kcal. With this enzyme, as with several others, kinetic studies were done to compare the 2 diastereomers of FGlu with Glu. With the exception mentioned for D-glutamate cyclase, Glu has a higher V_{max} value than either of the fluoroanalogues. Of the two fluoranalogues, generally the threo diastereomer is the preferred substrate.

α-Fluoro-γ-aminobutyric acid (FGABA) has been synthesized by the action of glutamate decarboxylase on FGlu; and this analogue of γ-aminobutyric acid (GABA) has been made available to several investigators studying the role of GABA in the central nervous system. It has been found by Professor L. L. Iversen that FGABA inhibits the physiological uptake of GABA in a brain preparation with an inhibitory constant which is comparable to the Michaelis constant for the uptake of GABA. In this system Professor D. R. Curtis has found that FGABA and FGlu have physiological actions which are more prolonged than the parent compounds in a spinal neuron preparation.

By tritium exchange it has been positive to prepare radioactive FGlu and to show that ^{3}M-FGlu can be incorporated into protein when it is added to the medium of wild type E. coli. Under the conditions employed, 1% Glu can be replaced by FGlu in E. coli protein.

Significance to Bio-Medical Research and the Program of the Institute:

The similarity in size between the carbon-fluorine bond and the carbon-hydrogen bond provides the basis of the action of two important metabolic inhibitors, e.g., 5-fluorouracil and fluoroacetate. 5-Fluorouracil has been used in chemotherapy of solid tumors, while fluoroacetate which leads to the lethal synthesis of fluorocitrate has been an important investigative aid for studying the effects in the organism of the selective inhibition of Krebs cycle. The introduction of fluorine for hydrogen has also been useful clinically e.g. 9α-fluorohydrocortisone. The stability of the carbon-fluorine bond prevents most of these compounds from undergoing biodegradation. Yet there is evidence that the carbon-fluorine bond may be broken in such important drugs as the anesthetic halothane. In this case the mechanism of carbon-halogen bond cleavage seems of particular importance since the question has been raised whether the alkylation which might accompany carbon-halogen bond cleavage could be responsible for the often fatal hepatoxicity of this widely used anesthetic. Thus with the increasing use of fluorinated compounds in clinical practice an understanding of the fundamental biochemistry of these compounds may offer an opportunity to avoid toxicity and extend the usefulness of certain drugs. In addition the usefulness of fluorinated analogs in both therapeutics and investigative biology suggests that a knowledge of the properties of the carbon-fluorine bond may provide a basis for the future development of other valuable drugs.

Proposed course:

The resolved isomers of FGlu and 3-fluoro-2-pyrrolidone-5-carboxylic acid will be examined by NMR and x-ray crystallography to see if these pyhsical methods will be useful in evaluating the structural differences between these analogues and their parent compounds. It may then be possible to make correlations between structural features and biochemical activity of these compounds.

Publications:

1. Unkeless, J. C., and Goldman, P.: Thermodynamic and Kinetic Aspects of the Reaction of γ-Fluoroglutamate with D-Glutamate Cyclase, J. Biol. Chem. (in press).

2. Unkeless, J. C., and Goldman, P.: The Diastereomers of γ-Fluoroglutamate: Complementary Structural Analogues, Mol. Pharmacol. (in press).

3. Kirk, K., and Goldman, P.: Fluorocitric Acid: Selective microbial degradation of the inhibitory isomer, Biochem. J. (in press).

Honors and Awards: None

Serial No. NIAMD-ARB-24c
1. Arthritis & Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Characterization of Previously Undescribed Nucleases which seem to be Residue-Specific

Previous Serial Number: NIAMD-ARB-22c

Principal Investigator: Dr. Peter Goldman

Other Investigators: Dr. Carl C. Levy (C-BC)

Cooperating Units: National Cancer Institute, Biometry Branch

Man Years:

Total:	0
Professional:	0
Other:	0

Proposed Course:

This project led to a publication and is now complete.

Publication:

Levy, C. C. and Goldman, P.: Residue-specificity of a ribonuclease which hydrolyses polycytidylic acid. J. Biol. Chem. 245:3257-3262, 1970.

Serial No. NIAMD-ARB-25c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Metabolic Actions of Salicylic Acid and Aspirin

Previous Serial Number: 23C

Principle Investigator: Dr. Peter Goldman

Other Investigators: Jay C. Unkeless

Cooperating Units: None

Man Years:

Total:	.8
Professional:	.5
Other:	.3

Project Description:

Objectives:

To study the metabolic alterations induced by salicylates and the reactions of salicylates which may be pertinent to their therapeutic efficacy.

Methods Employed:

An examination is being made of the chemical properties of salicylate and some of its analogues with a view toward establishing correlations that may explain the mode of action of this drug.

Possible intermediates in salicylate metabolism have been synthesized by organic chemical methods and some properties of these compounds have been studied.

Major Findings:

It has been reported that the Gravindex test for pregnancy can yield strong false positive reactions in patients taking salicylates. This finding seemed of particular interest since the phenomenon might serve as a useful model for considering the role of salicylates in modifying antigen-antibody reactions. Accordingly the Gravindex Test was performed on the urines of more than 30 patients receiving salicylate therapy on an out-patient basis

and 4 patients in the Clinical Center who had salicylate intake increased to the limit of toxicity. In this group only about 10% of the outpatient group had false positive reactions, an incidence comparable to that observed normally with the test. No positive reactions were found in the patients given the high doses.

The matter was further persued by examining the effect of salicylates and the metabolites of salicylates normally found in urine (salicylurate, gentisic acid, salicyl ether glucuronide) on the test when added to normal urine. None of the compounds had any effect on the test even when added at high concentrations. Because of these results the effect of salicylate on the Gravindex Test is no longer under study.

Significance to Bio-Medical Research and the Program of the Institute:

Salicylates are used for a variety of analgesic functions in addition to being a major therapeutic agent in rheumatoid arthritis. Their mode of action is largely unknown. Recently studies have raised the question of whether these compounds are teratogens. The lack of knowledge about these drugs which are used widely and over long periods of time suggests the need for further studies on them at a biochemical level. In addition if the mechanism of action of salicylate can be learned, the knowledge might be useful in the design of more effective therapeutic agents.

Proposed Course:

The chemical and biochemical effects of salicylates will be further studied in human and animal systems.

Honors and Awards: None

Publications: None

Section on Mineral Metabolism

The complete amino acid sequence of bovine parathyroid hormone has been deduced by sequential degradation of the native molecule as well as selected fragments. Extensive application was made of the automated Edman procedure for successive reaction of N-terminal amino acids with phenylisothiocyanate and cleavage of the amino-terminal residue by exposure to heptofluorobutyric acid. Succinylation of the 84-amino acid hormonal molecule allowed isolation of the C-terminal dotriacontapeptide. This fragment was sequenced by the automated Edman method applied to the entire fragment as well as to subfragments prepared with chymotrypsin or with trypsin digestion of the deblocked dotriacontapeptide.
[Drs. Niall, Keutmann, Potts, MGH, and Dr. Aurbach, NIAMD]

Earlier studies by our group indicated that the N-terminal 30 to 44 residues of parathyroid hormone contained the biologically active portion of the molecule. Deduction of the amino acid sequence of the hormone made it possible to project synthesis of a portion of the molecule which would be biologically active. Synthesis by a solid-phase method was successfully completed to produce the 34-amino acid fragment representing the N-terminal portion of the hormone molecule. The specific biological effects of this synthetic peptide on bone and kidney are qualitatively identical to those of the native hormone in classical bioassays in vivo and in vitro. Thus, essential requirements for the physiological actions of the peptide on both skeletal and renal tissue are contained within the 34 amino-terminal residues.
[Drs. Tregear, Keutmann, Niall and Potts, MGH, and Dr. Aurbach, NIAMD]

Still further support has been gained for the thesis that the mechanism of action of parathyroid hormone is mediated through activation of adenyl cyclase in bone and kidney and consequent rise in tissue concentration of 3',5'-AMP. Dibutyryl-cyclic 3',5'-AMP added to fetal bones in vitro produces biological effects qualitatively similar to those effected by parathyroid hormone. It was found that the action of dibutyryl-3',5'-AMP can be attributed to induction of a rise in endogenous tissue concentration of 3',5'-AMP itself. This increase in tissue concentration of the cyclic nucleotide is brought about by inhibiting cyclic nucleotide phosphodiesterase in the cell. Studies with tracer amounts of dibutyryl-3',5'-AMP proved that the concentration of the latter reached sufficient concentrations within the cell to effectively inhibit endogenous phosphodiesterase. Heretofore it had been thought that dibutyryl-3',5'-AMP acted with cells as an analogue of 3',5'-AMP by activating the same intracellular systems affected by the natural cyclic nucleotide.
[Drs. Heersche and Aurbach, NIAMD]

A means of stabilizing renal adenyl cyclase to prolonged storage was developed. Preparation of the enzyme in buffer containing 10% v./v. dimethylsulfoxide allows storage in liquid nitrogen for periods up to several months without significant loss of sensitivity to parathyroid hormone. This finding allowed the development of a highly sensitive and specific in vitro bioassay for parathyroid hormone. Extensive studies have been carried out on the kinetics of activation of the enzyme by fluoride or parathyroid hormone. Parathyroid hormone is fully active with Mg/ATP ratios of unity. Higher Mg/ATP ratios are required for activation by fluoride suggesting that there is an additional enzyme site for Mg which is exposed in the presence of fluoride.
[Drs. Marcus and Aurbach, NIAMD]

A protein kinase from bovine renal cortex has been identified and purified 10-fold. This enzyme catalyzes the ATP-dependent phosphorylation of arginine-rich histone and is activated by the addition of 3',5'-cyclic-AMP. The K_m for 3',5'-AMP in this reaction is approximately 10^{-8} M. The reaction requires magnesium although a slow rate of reaction can be obtained with manganese. Recent experiments with Biogel fractionation indicate that the 3'5'-AMP binding unit is separable from the kinase. Unlike results reported with other tissues, sucrose gradient ultracentrifugation did not afford separation of binding protein from kinase protein derived from renal cortex. This work should ultimately allow the identification of the physiological substrate phosphorylated in the kidney in response to parathyroid hormone.
[Drs. Winickoff and Aurbach, NIAMD]

Further studies have been carried out on clinical syndromes considered refractory to parathyroid hormone. Approximately eighteen cases of pseudo-hypoparathyroidism have now been examined and virtually without exception these subjects show a defective response to parathyroid hormone detected by analyzing for cyclic AMP in the urine. The hypothesis is that this disorder may be attributed to a genetically deficient or defective adenyl cyclase normally sensitive to parathyroid hormone in bone and kidney.
[Drs. Marcus, Winickoff, Marx and Aurbach, NIAMD]

We have extended these studies to test other clinical syndromes unrelated to parathyroid disorders that have appeared refractory to parathyroid hormone in tests for phosphaturia. These syndromes include Gardner's syndrome, basal cell nevus syndrome,syndrome of calcification of the basal ganglia, and vitamin D-resistant osteomalacia. Patients in these categories showed normal responses to parathyroid hormone as determined by excretion of 3',5'-AMP. It was concluded that the classical phosphaturic test is neither precise nor sensitive enough to discriminate normal subjects from those refractory to parathyroid hormone.
[Drs. Marcus, Winickoff and Aurbach, NIAMD, and Drs. Nigra and Epstein,NCI]

Section on Physiology and Clinical Nutrition

At the beginning of FY 1971, Dr. Jerry Gardner left SPCN for a post in DHDB, and was replaced as CA by Dr. Michael Droller. Dr. Sidney Wolfe returned from a year of advanced residency training at Case Western Reserve University.

Reinstrumentation of the Metabolic Chamber is proceeding apace, with the considerable help of a succession of college students, admitted to the CC as normal volunteers, who find an outlet for their interests in physics, electronics, and instrumentation by working in the chamber shop as a career assignment, under the direction of Dr. R. Thompson. Equipment for setting respiratory gas flow at desired levels is now being tested, and oxygen and carbon dioxide analyzers are workable. Testing of the ionization chamber for measuring radioactive carbon dioxide will begin soon. (Dr. R. Thompson)

The metabolic chamber itself continues to serve as a controllable environmental chamber for the study of environmental physiology in man. A major interest continues to be the metabolic and secretory processes in eccrine sweat glands. Using normal volunteers treated with tracer doses of radioisotopes, we have demonstrated that the large amount of lactic acid in sweat is produced by glandular metabolism, and that it is derived from the glucose, not the lactate, of blood. Studies with radioactive urea are now under way, the intent being to discover why the concentration of this substance in sweat so uniformly exceeds its concentration in plasma. Preliminary results appear to show that there is a slowly turning over pool of urea trapped in the skin at a concentration higher than that of blood, and that this urea diffuses into sweat as it traverses the epidermal portion of the duct. Synthesis of urea by sweat gland tissue seems not to play a role. (Drs. R. Gordon, R. Thompson)

Several members of a family with impaired temperature regulation are under study. The syndrome seen in this kindred is characterized by diabetes, progressive optic atrophy, and inability to sweat. The basic cause of the disorder remains unknown, but it appears that it may be identified as a third type of familial dysautonomia. Affected individuals, on exposure to dry heat, sweat very little, and their body temperature rises steadily. Further studies on the group are planned for the summer of 1971, when several affected children will be free from school. (Drs. S. Wolfe, R. Thompson, and J. Marquardt, NEI)

During his year away, Dr. Wolfe continued with investigations into the metabolic abnormalities associated with alcohol withdrawal syndromes. This work gave further support to the theory that many of the symptoms of acute withdrawal are mediated, at least in part, by the development of a respiratory alkalosis. Studies are now being initiated, in collaboration with the Phoenix Indian Medical Center, to evaluate the possible therapeutic potential of acetazolamide in this condition. (Drs. S. Wolfe, S. Grundy, W. Gough, and A. Metzger.)

In collaboration with members of CHB, Drs. Wolfe and Droller, and Mr. J. Muenzer are studying various aspects of the metabolism and function of human blood platelets. Work this year has shown that prostaglandin, theophylline, and dibutyryl cyclic AMP, all of which raise intracellular levels of cyclic AMP in platelets, inhibit platelet function (aggregation and the release reaction) and platelet energy metabolism (lactate production). As part of a study to investigate the extrapancreatic effects of tolbutamide, it has been found that this drug also inhibits platelet function as above. Furthermore, tolbutamide inhibits platelet phosphodiesterase activity. Although the effect on this enzyme would provide an attractive explanation for its inhibition of platelet function, studies thus far have failed to show that tolbutamide causes a rise in intracellular level of cyclic AMP. In studies using a Clark electrode, it has been shown quite clearly that thrombin induces a sudden burst in oxygen consumption by platelets. This burst is inhibited by prostaglandin and also by a number of compounds which affect oxidative phosphorylation. Studies to determine the extent and mechanism by which aspirin inhibits platelet function are in progress. Both collagen- and thrombin-induced release reactions are inhibited by concentrations of aspirin as low as 20 mg%. The temperature-dependence of this inhibition is currently under investigation.

Serial No. NIAMD-MDB-1 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in Bone Metabolism

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. Robert Marcus, Richard Winickoff, Stephen Marx, James Phang and G. Donald Whedon.

Cooperating Units: Dr. Mones Berman, Mathematical Research Branch, NIAMD
Metabolism Branch, NCI

Man Years:
Total: 2.50
Professional: .75
Other: 1.75

Project Description:

Objectives: 1) To investigate the factors influencing mineral storage and loss in demineralizing bone diseases. 2) To investigate the response of the parathyroid gland to rapid changes in blood calcium. 3) To study the influence of phosphate on resorption of bone mineral.

Methods Employed: Metabolic balance studies in patients with disorders of calcium metabolism, noting the effects on nitrogen, calcium, phosphate and magnesium balances of various dietary intakes of calcium, phosphate and magnesium. 2) Estimation of pool sizes, turnover rates, bone deposition rates, and absorption rates of calcium in patients by the oral and intravenous administration of tracer doses of ^{47}Ca.

Major Findings: Same as those reported 1968-'69 and 1969-'70.

Honors and Awards: None

Publications:

1. Whedon, G. D.: Symposium comment. In Barzel, U. S. (Ed.): Osteoporosis. New York, Grune & Stratton, Inc., 1970, pp. 266-272.

Serial No. NIAMD-MDB-2 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Study of Parathyroid Hormone: Physiological Regulation of Secretion; Physical, Chemical, and Immunochemical Properties; Biochemistry; and Structure, and Mechanism of Action.

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. Robert Marcus, Richard N. Winickoff, Stephen Marx, Johannes Heersche, John T. Potts, Jr., Geoffrey Tregear, Hugh Niall, G. P. Mayer, D. Kronfeld and C. Ramberg.

Cooperating Units: University of Pennsylvania School of Veterinary Medicine
Endocrine Unit, Massachusetts General Hospital

Man Years:

Total:	6.50
Professional:	2.75
Other:	3.75

Project Description:

Objectives: To prepare purified parathyroid hormone in pilot-plant quantities; to study chemical properties and structure of the hormone; to relate chemical properties and structure to biological activity and to study the mechanism of action of the hormone; to develop a clinically useful test for circulating parathyroid hormone; to study the physiological regulation of secretion of the hormone.

Methods Employed: Pure parathyroid hormone is prepared from phenol extracts of bovine parathyroid glands by solvent fractionation, gel filtration and chromatography on carboxymethylcellulose. Purity is assessed by disc gel electrophoresis, ultracentrifugation, immunodiffusion and immunoelectrophoresis, and recovery of the amino- and carboxyl-terminal amino acids. The hormone is assayed in vivo in rats, in vitro by measuring hormonal activation of adenyl cyclase, or by a highly sensitive radioimmunoassay. Specific chemical and enzymatic methods are used to cleave the hormone to smaller polypeptide fragments. The polypeptide derivatives are sequentially degraded with exopeptidases and the Edman reaction. Adenyl cyclase is measured by determining conversion of α-^{32}P-labeled ATP to

cyclic 3',5'-AMP. Endogenous concentration of cyclic 3',5'-AMP is determined by 1) converting the cyclic nucleotide to ATP and measuring the latter with a ^{32}P-ATP exchange reaction or 2) radioimmunoassay.

Major Findings: Mechanism of Action.- Our earlier studies showed that the parathyroid status of animals is an important factor regulating excretion of cyclic AMP in the urine. This finding led to development of the thesis that cyclic AMP is involved in the mediation of parathyroid hormone action. Subsequent work showed that parathyroid hormone in vitro activates adenyl cyclase in the kidney as well as in bone, the two organs known to be physiological receptors for action of the hormone. Adenyl cyclase in the kidney is found in two different anatomic zones; adenyl cyclase in the cortex responds specifically to parathyroid hormone whereas that in the medulla responds to vasopressin. Further studies show that virtually all the adenyl cyclase detectable in kidney tissue is found within the renal tubules. This work establishes that the receptor for parathyroid hormone in kidney resides within the renal cortical tubule as had been suspected but not proven by indirect in vivo physiological experiments.

A means of stabilizing renal adenyl cyclase to prolonged storage was developed. Preparation of the enzyme and buffer containing 10% v/v dimethylsulfoxide allowed storage in liquid nitrogen for periods up to several months without significant loss of activity or sensitivity to the hormone. This finding allowed the development of a highly sensitive and specific in vitro bioassay for parathyroid hormone. A log linear response to hormone is obtained in the range of 0.14 to 1.1 USP units of hormone. Kinetic studies with this preparation showed distinct differences between hormone- and fluoride-activated enzyme activity in terms of Mg requirements. Fluoride-activated enzyme required the greater ratios of Mg/ATP. This implied that an additional Mg site exists for the enzyme under conditions of fluoride activation.

Addition of parathyroid hormone to fetal rat calvaria incubated in vitro causes a 10-fold increase in the intracellular concentration of cyclic AMP within five minutes. Physiological changes in calcium release begin to occur 1 to 2 hours after the rise in intracellular 3',5'-AMP. Other studies show that dibutyryl-3',5'-AMP (DBC) added in vitro mimics the physiological effects of parathyroid hormone. We have now elucidated the mechanism through which the effects of DBC are produced. After adding dibutyryl 3',5'-AMP in vitro there was a rapid increase in concentration of endogenous 3',5'-AMP. This rise in tissue concentration of 3',5'-AMP can be accounted for by inhibition by DBC of cyclic nucleotide phosphodiesterase within the cell.

A protein kinase from bovine renal cortex has been identified and purified 10-fold. This enzyme catalyzes the ATP-dependent phosphorylation of arginine-rich histone and is activated by the addition of 3',5'-cyclic AMP. The K_m for 3',5'-AMP in this reaction is approximately 10^{-8} M. The reaction requires magnesium, although a slow rate of reaction can be obtained with manganese. Recent studies indicate that it may be possible to isolate the enzyme and this should allow ultimately the identification of the physiological substrate phosphorylated in the kidney and bone in response to parathyroid hormone.

Chemistry of Parathyroid Hormone.- Application of the "sequenator method" to analysis of parathyroid hormone has allowed deduction of the complete amino acid sequence of bovine parathyroid hormone. The molecule is an 84-amino-acid polypeptide with alanine as the amino-terminal residue and glutamine at the C-terminus. There are two phenyl alanines (residues 7 and 34), two methionines (residues 8 and 18), one tryptophan (23) and one tyrosine (43). Previous work from this laboratory showed that the biologically important region of the molecule was located at the amino terminus within a polypeptide smaller than that demarcated by the first aspartate residue. Deduction of the sequence of the molecule allowed chemical synthesis of an amino-terminal biologically active fragment. The tetratriacontapeptide representing residues 1-34 in the hormonal molecule was synthesized by a solid-phase method.

After deblocking and purification the synthetic peptide was tested for biological and immunological activity. The product showed qualitatively all the biological properties, in vivo as well as in vitro, of the native molecule. Thus, the protean effects of the hormone on both renal and skeletal tissue can be accounted for by the same limited region of the molecule. It is also implied that the receptor sites for the hormone must be similar in bone and kidney.

Physiology.- Further studies have been carried out on the regulation of secretion of parathyroid hormone. Tests on cows with "milk fever" or parturient paresis show that in secondary hyperparathyroidism secretion of hormone is still under the control of blood calcium. Graphical presentation of hormone secretory rates versus serum calcium show steeper slopes in secondary hyperparathyroidism than in normal animals. Thus, secondary hyperparathyroidism can be defined functionally as a state of augmented secretory response to physiologic stimulus. It is presumed that the augmented secretory response directly reflects the degree of glandular hyperplasia.

Human Parathyroid Hormone.- Parathyroid hormone was purified from human parathyroid adenomas. Earlier studies proved that the human hormone is different immunologically from the bovine hormone. Amino acid analyses of the hormone purified from each source indicate eight or nine amino acid substitutions in the human hormone. These changes are predominantly in the neutral amino acids. It is probable that any major substitutions occur outside the amino terminal region important for biological activity. Substitutions beyond this region undoubtedly account for the immunological distinctions between the hormones of different species.

A study was made of the immunological reactivity of human parathyroid hormone isolated from adenomas. By making use of two different antisera, it was possible to show that human parathyroid hormone is distinctly different in immunoreactivity from the bovine hormone. Under most ideal conditions in the immunoassay for bovine hormone, the human hormone shows only one-half the reactivity. These findings show the necessity for using a reference preparation of human hormone in assays for parathyroid hormone in human serum.

Significance to Bio-Medical Research and Program of the Institute: Same as for 1968-'69 and 1969-'70.

Proposed Course of Project: Same as for 1968-'69 and 1969-'70.

Honors and Awards: Dr. G. D. Aurbach was elected to the Association of American Physicians.

Publications:

1. Aurbach, G. D., Marcus, R., Heersche, J., Marx, S., Niall, H., Tregear, G., Keutmann, H. T., Deftos, L. J., and Potts, J. T., Jr.: Hormonal regulation of cyclic 3',5'-AMP in specific receptor tissues. Israel J. Med. Sci. 7: 341-342, 1971

2. Heersche, J. and Aurbach, G. D.: Mode of action of dibutyryl cyclic AMP on bone. Israel J. Med. Sci. 7: 344-346, 1971.

3. Niall, H., Keutmann, H., Sauer, R., Hogan, M., Dawson, B., Aurbach, G. D., and Potts, J. T., Jr.: The amino acid sequence of bovine parathyroid hormone I. Hoppe-Seyler's Zeitschrift für Physiologische Chemie 351: 1586-1588, 1970.

4. Potts, J. T., Jr., Tregear, G. W., Keutmann, H. T., Niall, H. D., Sauer, R., Deftos, L. J., Dawson, B. F., Hogan, M. L., and Aurbach, G. D.: Synthesis of a biologically active-N-terminal tetratriacontapeptide of parathyroid hormone. Proc. Nat. Acad. Sci. USA 68: 63-67, 1971.

Serial No. NIAMD-MDB-3 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Chemical Nature and Mode of Action of Thyrocalcitonin.

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Project temporarily discontinued.

Serial No. NIAMD-MDB-4 (c)
1. Metabolic Diseases Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Pseudohypoparathyroidism and Related Disorders

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. Robert Marcus, Richard N. Winickoff, Stephen Marx, Thomas P. Nigra and Ervin H. Epstein

Cooperating Units: National Cancer Institute

Man Years:
Total: 2.75
Professional: 1.25
Other: 1.50

Project Description:

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism." Patients with this disorder differ from those with idiopathic hypoparathyroidism in that they have characteristic constitutional features and they do not respond to exogenous parathyroid extract. Albright proposed that this disorder was attributable to lack of end organ sensitivity to normally-secreted endogenous parathyroid hormone. Our findings that parathyroid hormone action is likely mediated through activation of adenyl cyclase, led us to test cases of pseudohypoparathyroidism by giving intravenous parathyroid hormone and measuring cyclic 3',5'-AMP in the urine. Pseudopseudohypoparathyroidism is a variant of the disorder and may occur in the same families with pseudohypoparathyroidism. Pseudopseudohypoparathyroidism is characterized by similar constitutional features but no clinical evidence of hypoparathyroidism. Several other syndromes including Gardner's syndrome, basal cell nevus syndrome, syndrome of calcification of basal ganglia, and vitamin D-resistant osteomalacia have also been reported in the past as resistant to the phosphaturic action of parathyroid hormone. Current studies have been extended to include testing patients in these categories as well.

Methods: Parathyroid hormone purified in our laboratories was sterile-filtered for clinical use through courtesy of the N.I.H. Pharmacy. Radioimmunoassay of parathyroid hormone was carried out as described previously. Measurement of 3',5'-AMP was performed according to the procedure devised in this laboratory.

Major Findings: Approximately 70 cases of this disorder have been described in the literature; we have examined 18 of these to date. In normal subjects, parathyroid hormone causes a 30- to 60-fold rise in urinary excretion of cyclic AMP within 30 minutes or less after intravenous administration of the hormone. A similar response has been observed in idiopathic, hypoparathyroid, surgical hypoparathyroid, and pseudopseudohypoparathyroid subjects (the latter category show the constitutional changes but do not have laboratory evidence of hypoparathyroidism). Parathyroid hormone caused little or no increase in excretion of cyclic AMP in patients with pseudohypoparathyroidism. In one case, studied through the cooperation of a clinical investigator at another hospital, there was a moderate rise in excretion of cyclic AMP; however, the diagnosis in this instance may be incorrect, and as yet we have not had the opportunity of seeing the patient at the Clinical Center. Patients with this disorder who fail to respond to parathyroid hormone showed a similarly defective response after prolonged calcium infusion. Parathyroid hormone was detectable in the plasma of pseudohypoparathyroid subjects before infusion of calcium but not after. This experiment tended to rule out the possibility that an abnormal endogenously secreted hormone immunologically reactive but biologically inert interfered with the action of exogenous parathyroid hormone.

The best evidence to date is that pseudohypoparathyroidism represents a genetic defect inherited as a sex-linked, dominant trait. There have been no documented cases of direct male-to-male transmission of the disease and the sex incidence is approximately 2:1 female-to-male. Several pedigrees have been described wherein pseudohypoparathyroid subjects were progeny of pseudopseudohypoparathyroid parents or vice versa. One family studied at the N.I.H. included a mother with pseudopseudohypoparathyroidism and her daughter with pseudohypoparathyroidism. The daughter failed to respond to exogenous hormone but the mother responded normally. We have also observed that, although the response to pseudopseudohypoparathyroidism is normal to exogenous hormone, there is a significantly increased rate of baseline excretion of cyclic AMP in this group. This observation may be of importance in further delineation of the precise genetic mechanism involved in transmission of the disorder. It appears likely that the cause of the disorder may be the existence of genetically deficient or defective adenyl cyclase in the bone and kidney of these subjects.

There is an association of hypothyroidism with pseudohypoparathyroidism and in a few such cases the hypothyroidism has been attributed to selective deficiency of thyrotropin. It seems possible that hypothyroidism in pseudohypoparathyroidism might reflect an abnormal receptor adenyl cyclase complex in the central nervous system or anterior pituitary analogous to the defect in the receptor for parathyroid hormone in kidney and bone.

Three patients with coexistent hypothyroidism and pseudohypoparathyroidism were given thyrotropin-releasing hormone as part of a study designed to localize the site of the thyroid defect. One case showed an abnormally low basal concentration of thyrotropin in plasma, but gave a normal response to thyrotropin-releasing hormone. In the two other cases, a mother and daughter, there were high basal concentrations of thyrotropin in plasma. The mother was tested with TRH and showed the exaggerated response of TSH secretion characteristic of primary hypothyroidism. The defect in the first case appears to be at the level of the hypothalamus or higher. The defect in the second two cases may represent an abnormality of the thyroid receptor for TSH itself.

Patients with Gardner's syndrome, basal cell nevus syndrome, syndrome of calcification of the basal ganglia, and vitamin D-resistant osteomalacia showed normal responses to parathyroid hormone as determined by urinary excretion of 3',5'-AMP. It was concluded that the classical phosphaturic response to parathyroid hormone is neither precise nor sensitive enough to discriminate normal subjects from those refractory to parathyroid hormone.

Significance to Bio-Medical Research and Program of the Institute: Same as reported for 1968-'69 and 1969-'70.

Proposed Course of Project: Same as reported for 1968-'69 and 1969-'70.

Honors and Awards: None

Publications:

1. Aurbach, G. D., Marcus, R., Winickoff, R. N., Epstein, E. H., Jr., and Nigra, T. P.: Urinary excretion of 3',5'-AMP in syndromes considered refractory to parathyroid hormone. Metabolism 19: 799-808, 1970.

2. Aurbach, G. D.: Parathyroid hormone. In Beeson, P. B., and McDermott, W. (Eds.): Cecil-Loeb Textbook of Medicine, 13th edition. Philadelphia, W. B. Saunders Co., 1971, pp. 1846-1855.

3. Aurbach, G. D.: Osteodystrophy: biochemical defect in pseudohypoparathyroidism. In Bergsma, D. (Ed.): Birth Defects; Original Article Series, Vol. VI, No. 3. New York, The National Foundation-March of Dimes, 1970, pp. 24-25.

Serial No. NIAMD-MDB-5 (c)
1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Total Energy and Substrate Metabolism: Studies in Health and Disease.

Previous Serial Number: Same

Principal Investigator: Dr. Robert S. Gordon, Jr.

Other Investigators: Dr. Ronald H. Thompson

Cooperating Units: None

Man Years:
Total: 1.00
Professional: .50
Other: .50

Project Description:

Objectives: To apply the technique of continuous, extended indirect calorimetry (respiratory gas exchange) and a number of related metabolic and physiologic techniques to the clinical study of energy metabolism, a) to elucidate the mechanisms of normal physiologic response, particularly temperature regulation, to various stimuli including climatic stress, b) to study the influence and mechanism of action on metabolism of a variety of endocrine hormones and drugs, and c) to investigate the characteristics of energy expenditure, energy balance, and aberrations of metabolic processes in a variety of disease conditions.

Methods Employed: Indirect human calorimetry by means of complete continuous expired air analysis in the Metabolic Chamber, metabolic balance determinations, total body heat loss by thermoelectric and radiometric techniques under controlled environmental conditions, and precise measurement of evaporative and metabolic weight loss.

Accomplishments: Total reconstruction of the metabolic monitoring system has been completed and testing phases have begun. Preliminary tests show that the system will measure and record O_2 uptake and CO_2 output as accurately as originally planned, although a relatively extensive period of calibration will be required. Testing of the $^{14}CO_2$-sensitive ionization chamber is about to begin.

The environmental chamber has been used for continuation of the study of digital blood flow in patients with Raynaud's syndrome. This investigation has been expanded to include patients with cutaneous moniliasis (from NIAID). Although these two conditions have some superficial similarities, the studies showed clearly that patients with cutaneous moniliasis maintain acral blood flow in the cold.

The heat and cold stress capability of the chamber has been used to study thermoregulatory responses in two patients with fever of unknown origin (NIAID) and in five members of a kindred in which an apparently hereditary dysautonomia is associated with marked impairment of sight and hearing (NEI). Finally, the chamber has served extensively as a controlled hot environment for studies of sweat gland physiology reported elsewhere (NIAMD-MDB-9-c).

Other instrumentation projects have included the development of a more comfortable probe for the measurement of sweat rate from a small area of the body, and simultaneous recording of surface temperature from another point.

Significance to Bio-Medical Research and the Program of the Institute: Same as for 1969-1970.

Proposed Course of Project: Same as for 1969-1970.

Honors and Awards: None

Publications: None

Serial No. NIAMD-MDB-6 (c)
1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Study of an Unusual Syndrome, Dysautonomia

Previous Serial Number: None

Principal Investigator: Dr. Sidney M. Wolfe

Other Investigators: Dr. Michael J. Droller and Mr. Joseph Muenzer

Cooperating Units: Dr. J. Marquardt, National Eye Institute

Man Years:
Total : 2.00
Professional: 1.00
Other : 1.00

Project Description:

Studies are in progress on several members of a family with a syndrome including diabetes, progressive optic atrophy, and heat intolerance. It appears that two siblings with this syndrome and at least one related adult have a temperature-regulatory disorder wherein their body temperature rises steadily when they are exposed to a hot environment. Thus far, it appears that they represent a third type of familial dysautonomia.

Significance to Bio-Medical Research and Program of the Institute: Previous studies demonstrated the presence of a new type of dysautonomia (type II) in two siblings. These children were unable to regulate their body temperature when placed in either warm or cold environment. The current studies involve what seems to be yet another type of familial dysautonomia which, although differing in other clinical respects, is similar to type II in that these children are also unable to sweat sufficiently to prevent their body temperature from rising when they are placed in a warm room. It appears that this disorder may differ from the type II in that the children respond to intradermal injections of pharmacological agents while sweating.

Proposed Course of Project: Further studies will be performed on the members of this family this summer when the two children are out of school in order to understand the nature of their inability to sweat.

Honors and Awards: None

Publications: 1. Wolfe, S. M., and Henkin, R. I.: Absence of taste in type II familial dysautonomia: unresponsiveness to methacholine despite the presence of taste buds. J. Pediatrics 77: 103-108, 1970.

Serial No. NIAMD-MDB-7 (c)
1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Metabolic Studies During Alcohol Withdrawal

Previous Serial Number: NIAMD-MDB-12 (c)

Principal Investigator: Dr. Sidney M. Wolfe

Other Investigators: Drs. S. Grundy, W. Gough, and A. Metzger, NIAMD Metabolic Research Division, U.S. PHS Indian Hospital, Phoenix, Arizona

Cooperating Units: None

Man Years
Total : .25
Professional: .25
Other : .00

Project Description:

Studies completed July 1970 in Cleveland (during year away from N.I.H.) gave further evidence of existence of respiratory alkalosis in all patients during alcohol withdrawal. Studies are in progress in cooperation with the NIAMD Metabolic Research Division at Phoenix Indian Hospital to study the efficacy of acetazolamide in the treatment of the alcohol withdrawal syndrome.

Significance to Bio-Medical Research and Program of the Institute: The appearance of significant respiratory alkalosis as early as 8 hours after alcohol withdrawal provides a possible explanation for the early withdrawal symptoms which often cause alcoholics to keep drinking. These metabolic aspects of withdrawal have not been previously documented and raise the question whether agents modifying acid-base balance may be useful in treating withdrawal.

Proposed Course of Project: Dr. Wolfe will continue a collaborative project with the NIAMD Metabolic Research Division, U.S. Public Health Service Indian Hospital in Phoenix. Alcoholics in withdrawal will be admitted to a metabolic ward and further studies with acidifying agents will be carried out.

Honors and Awards: None

Publications: None

Serial No. NIAMD-MDB-8 (c)
1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Platelet Metabolism

Previous Serial Number: None

Principal Investigator: Dr. Michael J. Droller

Other Investigators: Dr. Sidney M. Wolfe and Mr. Joseph Muenzer

Cooperating Units: Clinical Hematology Branch, NIAMD

Man Years:
Total : 1.75
Professional: 1.00
Other : .75

Project Description:

Further studies on platelet metabolism have shown that prostaglandin, theophylline, and dibutyryl cyclic AMP, all of which raise intracellular levels of cyclic AMP in platelets, inhibit platelet function (aggregation and the release reaction) and platelet energy metabolism (lactate production). As part of a study to investigate the extrapancreatic effects of tolbutamide, it has been found that this drug also inhibits platelet function as above. Furthermore, tolbutamide inhibits platelet phosphodiesterase activity. Although the effect on this enzyme would provide an attractive explanation for its inhibition of platelet function, studies thus far have failed to show that tolbutamide causes a rise in intracellular level of cyclic AMP.

In studies using a Clark electrode it has been shown quite clearly that thrombin induces a sudden burst in oxygen consumption by platelets. This burst is inhibited by prostaglandin and also by a number of compounds which affect oxidative phosphorylation.

Significance to Bio-Medical Research and Program of the Institute and Proposed Course of Project: Little information is available about the metabolism or physiology of the platelet within the first few second after interaction with thrombin, collagen or other aggregating agents. Studies to determine the extent and mechanism by which aspirin inhibits platelet function are in progress. Both collagen- and thrombin-induced release reactions are inhibited by concentrations of aspirin as low as 20 mg %. The

temperature-dependence of this inhibition is currently under investigation.

Honors and Awards: None

Publications:
1. Wolfe, S. M., and Shulman, N. R.: Inhibition of platelet energy production and release reaction by PGE_1, theophylline and cAMP. Biochem. Biophys. Res. Commun. 41: 128-134, 1970.

Serial No. NIAMD-MDB-9 (c)
1. Metabolic Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanisms of Secretion in Human Eccrine Sweat Glands

Previous Serial Number: NIAMD-MDB-7 (c)

Principal Investigator: Dr. Robert S. Gordon, Jr.

Other Investigators: Dr. Ronald H. Thompson and Mr. Del Thrasher

Cooperating Units: None

Man Years:
Total : 1.50
Professional: 1.25
Other : 1.25

Project Description:

Human sweat glands are minute, but have an extraordinarily high secretory capacity compared to their own weight. Therefore, the sweat gland may be a useful model system in the continuing effort to relate cellular metabolism to mechanisms of secretion. In addition, detailed knowledge of the function of sweat glands may be useful in "human engineering," and in elucidating those diseases, like cystic fibrosis, which affect sweat gland function.

Methods and Major Findings: During this year studies have centered on the use of internally administered radioisotopes to measure the rate of conversion and/or excretion of substrate from blood into sweat. After the test material has been administered, the subject lies for 1 to 3 hours on a hammock, and is exposed to a hot, dry environment (standard conditions 120°F, 12% relative humidity). Weight loss is recorded by the Brookline metabolic balance described in detail last year. At the end of the sweating period, solutes are collected by total body washdown. This wash water is then concentrated and subjected to chemical and radioisotopic analyses.

Comparing the amount of ^{14}C lactate found in sweat after the intravenous administration of either ^{14}C lactate or ^{14}C glucose enabled us to demonstrate that blood glucose is the direct metabolic precursor of sweat lactate. Radioactive lactate in the blood did not appear directly in sweat in detectable amounts. The radioactivity appearing in sweat after intravenous administration of labeled lactate could be accounted for entirely by its conversion back to glucose through the Cori cycle.

Investigation of the excretion in sweat of ^{14}C urea is now underway. Preliminary results indicate that, contrary to many previous theories, blood urea is not concentrated as it passes into sweat. On the contrary, urea coming from the blood is diluted as it enters the sweat by other urea previously stored in some pool, probably within the epidermis. Two to four days after the administration of labeled urea, when the radioactivity of circulating urea has largely died away, the specific activity of sweat urea is several times that of urea in the blood.

Proposed Course of Project: During the coming year a larger number of individuals will be studied with respect to the transport of labeled urea. We also anticipate doing similar studies with some other labeled non-electrolytes, most particularly ^{14}C mannitol, with which one or two experiments have already been carried out. The uptake of glucose by the sweat gland continues to be of interest. We hope to have the opportunity to investigate this further using isolated tissues in vitro, but this will depend on the availability of personnel skilled and interested in micro-dissection. It is our hypothesis that sweat gland tissue, like the central nervous system, is relatively independent of insulin for the uptake of glucose from the surrounding medium. We propose to test this, insofar as possible, in intact humans who either have diabetes, or who are rendered transiently similar to diabetics by means of a 72-hour fast.

Significance to Bio-Medical Research and the Program of the Institute: The processes whereby exocrine glands elaborate and transport their secretory products are of very general interest and are still imperfectly understood. It is hoped that our studies will throw some additional light on the secretory mechanism within the sweat gland, which may in turn be useful in interpreting the process of secretion in general. More particularly, since cystic fibrosis, a major area of program concern to NIAMD, is clearly manifest as an abnormality of sweat secretion, we always consider the possibility that further progress in understanding the secretion of normal sweat glands may throw light on the basic inherited defect of cystic fibrosis.

Honors and Awards: None

Publications:

1. Wolfe, S., Cage, G., Epstein, M., Tice, L., Miller, H., and Gordon, R. S., Jr.: Metabolic studies of isolated human eccrine sweat glands. J. Clin. Invest. 49:1880-84,1970.

2. Cage, Gary W., Wolfe, Sidney M., Thompson, Ronald H., and Gordon, Robert S., Jr.: Effects of water intake on composition of thermal sweat in normal human volunteers. J. Appl. Physiol. 29:687-690, 1970.

The work of this Branch is directed toward understanding the origin and treatment of human disease by examining derangements of underlying processes of metabolism and physiology. Because the digestive tract lends itself particularly well to the pursuit of this goal, and because this organ system permits one to extend animal studies into man, gastroenterology is the primary focus of the Branch's clinical and laboratory activities. During the past year increased emphasis was placed on studies of transport mechanisms in various tissues, with the intention of applying information gained from studies of such cells as erythrocytes to transport problems in the gastrointestinal tract. Earlier studies of inborn errors of metabolism, and the clinical disturbances they produce, were continued too.

Studies of the small intestine

We showed previously that when patients with gluten-sensitive enteropathy are challenged with dietary gluten, specimens of jejunal mucosa exhibit enhanced incorporation of ^{14}C-leucine into IgA and IgM. These earlier studies were based on immunoprecipitation techniques. During the past year a new method was developed based on solid-phase immunoadsorption techniques. The earlier findings were confirmed, and the work was extended to show that a significant fraction of the increased synthesis of IgA and IgM is attributable to the formation of antibodies to gluten. These findings greatly strengthen the hypothesis that an immunological mechanism is intimately involved in the pathophysiology of gluten-sensitive enteropathy. (Drs. Z. Myron Falchuk, Warren Strober and Leonard Laster)

Our study of factors involved in the regulation of the hydrolytic enzyme activities in the intestinal mucosa was continued. Biopsy specimens were maintained in organ culture for at least three days, and we showed that in man, after exposure of a biopsy specimen to hydrocortisone intestinal brush border lactase activity increases about two-fold at the end of 72 hours. None of the other enzymes assayed, namely sucrase, trehalase and alkaline phosphatase, exhibit such a rise. In a patient with primary intestinal lactase deficiency hydrocortisone failed to stimulate lactase activity *in vitro*. This system of organ culture may well lend itself to an exploration of the mechanisms by which hydrocortisone produces a change in the enzyme content of intestinal tissue. (Drs. Michael S. Brown, Saul G. Agus and Leonard Laster)

A comprehensive review was written on the digestive and transport functions of the columnar epithelial cell of the small intestine. A general summary of current hypotheses of membrane transport was followed by detailed comments on hexose and amino acid absorption, and on studies related to the molecular components of transport. The digestive processes of the columnar cell were reviewed in detail, some of the inborn errors of metabolism associated with these functions were discussed, and, finally, current information on the pathogenesis of celiac sprue was recounted. (Drs. Jerry D. Gardner, Michael S. Brown and Leonard Laster)

Studies on homocystinuria

In examining the effects of pyridoxine on patients with homocystinuria due to cystathionine synthase deficiency we found, as have others, that some patients with the disease respond to pyridoxine by a marked increase in urinary excretion of homocystine and a marked decrease in the concentration of this compound in blood. It is hoped that this effect will be therapeutic and our study, described in our previous report, tends to support that possibility.

During the past year we turned to the problem of those patients who do not respond to pyridoxine in this way. We showed that when patients with homocystinuria are given α-aminoisobutyric acid, a compound which appears not to be metabolized in man but to be excreted virtually entirely in the urine, the urinary excretion of homocystine in these patients increases greatly. Urinary excretion of glycine, arginine, lysine and ornithine also increases. The administration of α-aminoisobutyric acid to a normal patient is not followed by detectable urinary excretion of homocystine. This approach to the elimination of homocystine from the body of patients with cystathionine synthase deficiency may have therapeutic implications and it will be explored further. (Z. Myron Falchuk, William A. Edwards and Leonard Laster)

We have continued our exploration of the biochemical changes associated with homocystinuria, and have shown that patients with cystathionine synthase deficiency accumulate S-adenosyl homocystine in the liver, whereas patients without the disease do not have detectable amounts of this compound in their liver. This observation is consistent with current concepts of homocystine metabolism in man. Since homocystine accumulates in patients with cystathionine synthase deficiency, and since the equilibrium of the reaction in the transsulfuration pathway which leads to homocystine formation strongly favors the synthesis of S-adenosyl homocystine from homocystine and adenosine, this observation, one not hitherto recorded, is consistent with our concept of the disorder. (S. Harvey Mudd, William A. Edwards and Leonard Laster)

Membrane transport

Our studies of the transport of various substrates across cell membranes are directed toward characterizing the basic features of the transport process and investigating diseases in which altered membrane transport is a significant pathophysiologic or etiologic feature. In cystic fibrosis a "factor" (present in saliva and serum) has been suggested to be responsible for the abnormal ionic composition of the exocrine secretions in this disease. All attempts by this laboratory to demonstrate an effect of the "factor" on cation transport in non-exocrine tissues have been unsuccessful. Studies of sodium transport in erythrocytes from patients with Bartter's syndrome and from patients with Liddle's syndrome indicate that both disorders may represent a primary abnormality of membrane cation transport. (Drs. Jerry D. Gardner, Shlomo Shibolet, Artemis Simopoulis, Lynn Taussig and Roger Snyder)

Studies of the transport of dibasic amino acids in erythrocytes from normal subjects and from patients with cystinuria indicate that dibasic amino acids cross the erythrocyte membrane via two saturable mechanisms operating in parallel and that neither of these erythrocyte transport mechanisms is affected by the genetic defect in cystinuria. (Drs. Jerry D. Gardner, Arnold Levy and Leonard Laster)

Administrative activities

Dr. Laster's temporary assignment to the President's Office of Science and Technology continues. He assists Dr. Edward E. David, Jr., the Science Advisor to the President, in policy decisions related to biomedical research and health.

Serial No. NIAMD-DHDB-1(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of the small intestine

Previous Serial Number: SAME

Principal Investigator: Dr. Leonard Laster

Other Investigators: Drs. Saul G. Agus, Michael S. Brown, Z. Myron Falchuk, Jerry D. Gardner and Peter M. Loeb, NIAMD; Dr. Warren Strober, NCI

Cooperating Units: National Cancer Institute

Man Years:

Total:	2.5
Professional:	1.5
Others:	1.0

Project Description:

Objectives:

The purpose of this study is to investigate 1) intermediary metabolism of the mucosa of the small intestine, 2) factors which regulate mucosal metabolism, and 3) effects of disease on these metabolic processes.

Methods Employed:

Various techniques for studying tissue metabolism were employed. These included the use of isotopically labeled compounds in vitro and in vivo, and methods pertinent to enzymology and immunology.

Patient Material:

For the studies of immune mechanisms in gluten-sensitive enteropathy: 20 normal volunteers, 5 patients with gluten-sensitive enteropathy, 2 patients with malabsorption due to other causes, and 8 patients with immune deficiency diseases were studied. For the investigation of the regulation of mucosal enzyme activity: 5 normal volunteers and 1 patient with intestinal lactase deficiency were studied.

Major Findings:

1. Immune mechanisms in gluten-sensitive enteropathy. (Z. Myron Falchuk, Warren Strober, Peter M. Loeb and Leonard Laster)

This project is an extension of studies originally begun to examine protein synthesis in the human small intestine mucosa. During the past year new methods were developed for determining the synthesis of immunoglobulins by human intestinal mucosa in vitro, and these techniques were applied to an exploration of immune mechanisms in gluten-sensitive enteropathy. Last year a solid-phase immunoadsorbant assay was developed for IgA, and this year a similar method was developed for IgM. The synthesis of these immunoglobulins by jejunal biopsy specimens in vitro was studied with normal volunteers and patients with: a) sprue due to gluten-sensitive enteropathy, b) malabsorption in the absence of gluten-sensitive enteropathy, c) various immune deficiency states of the humoral variety, and d) cystic fibrosis and chronic pancreatitis.

By use of the new method, we confirmed our earlier findings (based on an immunoprecipitation technique) that challenging patients with gluten-sensitive enteropathy by exposure to a gluten-containing diet increases the production of IgA and IgM in vitro by intestinal mucosa.

A major aspect of this year's effort was devoted to determining whether the newly synthesized immunoglobulins include antibodies to gluten. We used an affinity chromatography technique in which the components of a gliadin digest were linked by covalent coupling to Sepharose. We showed that after patients with gluten-sensitive enteropathy were exposed to gluten, their intestinal mucosal specimens synthesized antibodies to gluten. When such patients were in remission, on a gluten-free diet, we could not detect synthesis of anti-gluten antibodies. Normal volunteers challenged with gluten did not produce anti-gluten antibodies. The antibodies produced by the patients were shown to be in the IgA and IgM classes.

These observations strengthen the hypothesis that an immunological mechanism is intimately involved in the pathophysiology of gluten-sensitive enteropathy. The next phase in this study will be directed toward establishing an in vitro system to test the pathogenecity of anti-gluten antibodies.

2. Regulatory mechanisms in the human small intestine mucosa. (Drs. M. Brown, Saul G. Agus and Leonard Laster)

The study described in last year's report was continued by obtaining intestinal biopsies from normal volunteers and maintaining the tissue in organ culture for periods up to 72 hours. We made serial determinations of intestinal brush border hydrolases, and found that when hydrocortisone is present in the incubation medium, intestinal brush border lactase activity doubles at the end of 72 hours. No such rise was seen with the

other enzymes studied, namely, sucrase, trehalase, and alkaline phosphatase. Tissue from a patient with intestinal lactase deficiency was studied under comparable conditions and hydrocortisone failed to induce an increase in lactase activity in that tissue.

3. Comprehensive review of intestinal microvillus membrane structure and function (Drs. Jerry D. Gardner, Michael S. Brown and Leonard Laster)

By invitation, a review was written about the transport and digestive functions of the intestinal microvillus membrane.

Significance to Biomedical Research and the Program of NIAMD:

These studies provide information about the role of local intestinal immune mechanisms in the pathophysiology of celiac sprue, and about the regulation of the biochemical activities of the intestinal mucosa.

Proposed Course:

These projects will be continued and the findings explored further.

Honors and Awards:

The study of immune mechanisms in gluten-sensitive enteropathy was accepted for presentation at the plenary session of the Annual Meeting of the American Federation for Clinical Research on May 2, 1971. The abstract was entitled: "Gluten-sensitive enteropathy--intestinal synthesis of anti-gluten antibodies in vitro," by Drs. Falchuk, Laster and Strober (Clin. Res. 19: 390, 1971).

Dr. Laster has continued his temporary assignment as a member of the President's Office of Science and Technology. He is responsible for the development of policy related to biomedical research and health, and assists the science advisor to the President.

Publications:

Loeb, P. M., Strober, W., Falchuk, Z. M., and Laster, L.: Incorporation of L-leucine-^{14}C into immunoglobulins by jejunal biopsies of patients with celiac sprue and other gastrointestinal diseases. J. Clin. Invest. 50: 559-569, 1971.

Gardner, Jerry D., Brown, Michael S., and Laster, Leonard: The columnar epithelial cell of the small intestine: digestion and transport. New Engl. J. Med. 283: 1196-1202, 1264-1271, 1317-1324, 1970.

Laster, L.: Diseases of the small intestine: Malabsorption syndrome. In Keefer, C. S. and Wilkins, R. W. (Eds.): Medicine: Essentials of Clinical Practice. Boston, Little, Brown Co., 1970, pp. 490-495.

Laster, L.: The small intestine. In Roe, F. J. C. (Ed.): Metabolic Aspects of Food Safety. Oxford and Edinburgh, Blackwell Scientific Publications, 1970, pp. 17-36.

Serial No. NIAMD-DHDB-2(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biophysical approaches to the study of human disease

Previous Serial Number: SAME

Principal Investigator: Dr. Leonard Laster

Other Investigators: Dr. A. J. Tousimis, Biodynamics Research Corp. and Dr. Saul G. Agus, NIAMD

Cooperating Units: Biodynamics Research Corp. (Contract #PH 43-66-10)

Man Years:

Total:	2
Professional:	1
Others:	1

Project Description:

Objectives and Methods:

A collaborative study is in progress with Dr. A. J. Tousimis for the purpose of applying biophysical techniques to the study of biological problems. At this time we are using the small intestine as a model tissue. The techniques involved include high resolution electron microscopy, electron probe microanalysis, and scanning electron microscopy. This work is sponsored under NIH Contract #PH 43-66-10 with Biodynamics Research Corporation ($44,350 for FY 1971).

Patient Material:

Tissue is submitted from all patients with small bowel disease who undergo peroral biopsies.

Major Findings:

1. As our ability to undertake a quantitative analysis of biological specimens for at least 9 elements improves, we are accumulating data on the ionic content of thin frozen-dried sections of hamster liver, hamster intestine, and human intestinal biopsies.

2. We are continuing studies of the use of the electron probe to identify the general nature of the compounds in which sulfur is detected in various tissues. We are making use of the differences in the X-ray line produced by a given element as detected in the probe when it is embodied in different compounds. We have applied this technique to the study of patients with Wilson's disease, and have evidence that the copper exists in a complex with a sulfur-containing protein.

3. We are studying the use of combined scanning and transmission electron microscopy in the examination of biological tissue specimens. Biopsy specimens from the human small intestine are examined directly in the scanning electron microscope, and information about the cell surface and internal structure is obtained.

Significance to Biomedical Research and the Program of NIAMD:

These studies are designed to provide insight into the structure and chemical composition of intestinal mucosa in health and disease.

Proposed Course:

If support for Contract #PH 43-66-10 is continued, all of these studies will be developed further.

Honors and Awards:

None

Publications:

None

Serial No. NIAMD-DHDB-3(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemical aspects of disease

Previous Serial Number: SAME

Principal Investigator: Dr. Leonard Laster

Other Investigators: Drs. Saul G. Agus and Z. Myron Falchuk, NIAMD, Mr. William A. Edwards, NIAMD and Dr. S. Harvey Mudd, NIMH

Cooperating Units: National Institute of Mental Health

Man Years:

Total:	2.5
Professional:	1.5
Others:	1.0

Project Description:

Objectives:

The purpose of this study is to examine pathways of metabolism in tissues of the gastrointestinal tract and the disruption of these pathways in disease.

Methods Employed:

Studies of homocystinuria required extensive use of the amino acid analyzer.

Patient Material:

Investigations of homocystinuria: 1 normal volunteer and 2 patients with proven cystathionine synthase deficiency were studied. In addition, specimens of liver tissue were obtained from 6 patients without cystathionine synthase deficiency, and from 3 patients with the disease.

Major Findings:

1. Cystathionine synthase deficiency (homocystinuria)--effect of α-aminoisobutyric acid (AIB) on urinary excretion of homocystine (Dr. Z. Myron Falchuk, Mr. Wm. A. Edwards and Dr. Leonard Laster)

The administration of pyridoxine to some but not all patients with homocystinuria is followed by a marked increase in the urinary excretion of homocystine and a marked diminution in the concentration of homocystine in their blood. Presumably pyridoxine exerts a therapeutic or salutary effect on the patients. In an effort to study patients who do not respond in such a manner to the administration of pyridoxine, we explored the possibility of producing an increased urinary excretion of homocystine by the use of an agent that would compete with homocystine for its renal transport mechanism. In previous studies in this laboratory (Laster and Matthews) it was found that the non-metabolizable amino acid, α-aminoisobutyric acid (AIB), produces an aminoaciduria in normal subjects. We therefore tested the effect of AIB on two patients with homocystinuria. Each was given a short course of treatment with AIB and the pattern of excretion of urinary amino acids was studied before and after administration of the compound. One normal volunteer was studied as a control subject. In the homocystinuric patients, AIB greatly enhanced the urinary excretion of homocystine, and of glycine, arginine, lysine and ornithine. The effect on the normal volunteer was variable, but there was a tendency toward an increased excretion of arginine, lysine and ornithine. Our data suggest that a common transport mechanism may exist for homocystine, arginine, ornithine, lysine and AIB in the human kidney, and that competition between AIB and homocystine at the renal level may account for the effect of AIB on homocystine excretion. The use of AIB or perhaps other analogs, may be of therapeutic importance in the management of patients with homocystinuria who do not respond to pyridoxine therapy.

2. Cystathionine synthase deficiency (homocystinuria)--tissue accumulation of adenosyl homocystine (Dr. S. Harvey Mudd, Mr. William A. Edwards and Dr. Leonard Laster)

We showed previously that homocystinuria is due to a deficiency of cystathionine synthase activity which results in the accumulation of homocystine. Homocystine is generated in the transsulfuration pathway by the enzymatic cleavage of S-adenosyl-homocystine to produce adenosine and homocystine. Since the equilibrium of this reaction strongly favors the formation of S-adenosyl-homocystine, it seemed reasonable that S-adenosyl-homocystine should accumulate in patients with cystathionine synthase deficiency. This question had not been explored previously. Methods were therefore developed to extract the amino acids from liver specimens and to determine whether S-adenosyl-homocystine was present in normal tissues or in tissues from patients with cystathionine synthase deficiency. We detected no S-adenosyl-homocystine in liver specimens from 6 patients without cystathionine synthase deficiency. However, liver specimens from each of 3 patients with the disease were indeed found to contain S-adenosyl-homocystine. This is the first demonstration of the accumulation of this compound in the disease. The observation further extends our understanding of the pathophysiology of this inborn error of metabolism.

3. Hepatitis and agammaglobulinemia (Drs. Saul G. Agus and Leonard Laster)

Some years ago one of our patients with hypogammaglobulinemia developed classical signs and symptoms of infectious hepatitis. The disease ran a benign course and she appeared to recover completely. During the past year a second patient was studied. She has severe hypogammaglobulinemia. She developed hepatitis associated with Australia antigen and her clinical course was a particularly severe one. In an effort to treat her, we infused high titre antibody to Australia antigen and made serial observations on antigen antibody titres and complement levels. She survived, and is regarded as unusual in having done so, since the few comparable patients seen elsewhere died.

Significance to Biomedical Research and the Program of NIAMD:

These studies extend our insight into approaches to the therapy of cystathionine synthase deficiency and deepen our understanding of the pathophysiology of the disease.

Proposed Course:

The general approach exemplified by these studies will be continued.

Honors and Awards:

None

Publications:

Mudd, S. H., Edwards, W. A., Loeb, P. M., Brown, M. S. and Laster, L.: Homocystinuria due to cystathionine synthase deficiency: the effect of pyridoxine. J. Clin. Invest. 49: 1762-1773, 1970.

Volpe, J. J., Lee, G., Laster, L., and Robinson, J. C.: Regional distribution of isozymes of D-amino-acid oxidase and acetylesterase in developing primate brain. Exptl. Neurol. 28: 76-87, 1970.

Serial No. NIAMD-DHDB-4(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of membrane transport in mammalian tissues

Previous Serial Number: NIAMD-MDB-13(c), NIAMD-MDB-6(c), NIAMD-MDB-11(c)

Principal Investigator: Dr. Jerry D. Gardner

Other Investigators: Drs. Shlomo Shibolet, Artemis Simopoulos, Lynn Taussig, Roger Snyder, Arnold Levy and Leonard Laster

Cooperating Units: Metabolic Diseases Branch, NIAMD
Endocrinology Branch, NHLI
Pediatric Metabolism Branch, NIAMD
Laboratory of Neurochemistry, NINDB

Man Years:

Total:	1.0
Professional:	0.5
Other:	0.5

Project Description:

Objectives:

1) To characterize functionally the mechanisms by which various substrates cross the plasma membrane of different mammalian cells; 2) to identify the metabolic and humoral factors which influence the transport of various substrates across the plasma membrane; 3) to develop techniques which will distinguish between binding of the substrate to the membrane and translocation of the substrate across the membrane.

Methods Employed:

By using slices of intact tissue, isolated cells or isolated plasma membranes and by using radioactive tracers, we studied the transmembrane movement of various substrates. Metabolism of the transported substrate was evaluated by use of techniques appropriate to the substrate being studied.

Major Findings:

1. Sodium transport in erythrocytes (Drs. Jerry D. Gardner, Shlomo Shibolet and Artemis P. Simopoulos)

In human and rabbit erythrocytes sodium content was measured flame photometrically. Sodium fluxes were measured using ^{24}Na. The effect of alterations in the cation composition of the incubation medium and ouabain (10^{-4} M) or ethacrynic acid (10^{-3} M) on sodium influx and sodium outflux were measured. Cation binding was evaluated using equilibrium dialysis. ATPase activity was determined in erythrocyte membranes by measuring ^{32}P liberation from gamma-^{32}P-ATP.

In human erythrocytes the kinetics of sodium influx and of sodium outflux can be explained in terms of a "carrier" having two sites. The two sites have different affinities for sodium ions and the rate of movement of the sodium-carrier complex across the membrane differs depending on which site is occupied. Potassium ions compete with sodium ions at the site having the greater affinity for sodium ions, but not at the site having a low affinity for sodium ions.

Sodium transport in rabbit erythrocytes can be explained by a similar mechanism. Compared to human erythrocytes, each of the two sites has a greater affinity for sodium ions and the affinity of the high-sodium site for potassium is decreased. In addition, the calculated number of carriers per cell in rabbit erythrocytes is approximately 4-5 times greater than that in human erythrocytes.

2. Amino acid transport in human erythrocytes (Drs. Jerry D. Gardner, Arnold Levy, and Leonard Laster)

In human erythrocytes we measured the influx, outflux and steady--state concentration of ^{14}C-dibasic amino acids *in vitro*.

The major features of dibasic amino acid transport in human erythrocytes are that it is temperature-dependent, incapable of uphill transport, not dependent on extracellular sodium or potassium concentration or on energy derived from cellular metabolism. Kinetic studies show that lysine transport in human erythrocytes comprises two saturable ("carrier--mediated") processes operating in parallel: one is a high-affinity, low-capacity process which predominates at low lysine concentration; the other is a low-affinity, high-capacity process which predominates at higher lysine concentrations.

3. Cation transport in cultured brain cells (Drs. Jerry D. Gardner, Shlomo Shibolet and Roger Snyder)

Stable lines of the following cell types have been maintained in tissue culture: Fetal hamster astrocytes, fetal hamster oligodendrocytes, astrocytes derived from a human astrocytoma, oligodendrocytes derived from a human oligodendroglioma, and a mixed cell population derived from a human neuroblastoma. Cell volume was determined gravimetrically. Sodium and potassium content were measured with a flame photometer. Sodium and potassium fluxes were measured with ^{24}Na and ^{42}K. Cation binding was measured by equilibrium dialysis.

All of the above cell types were found to have high potassium and low sodium contents. Equilibrium dialysis studies using a "plasma membrane fraction" demonstrated significant binding of sodium but not potassium by human astrocytes. In contrast to the mixed cell population, human astrocytes showed a significant increase in cell volume following exposure to an elevated potassium concentration. Potassium transport kinetics indicate the presence of two intracellular compartments whose turnover times differ by at least one order of magnitude.

During the past six months this project was temporarily suspended because of Dr. Snyder's absence from NINDB. Tentative plans are to resume these studies upon Dr. Snyder's return.

4. Membrane transport in the small intestine (Drs. Jerry D. Gardner, Lynn Taussig and Leonard Laster)

During this past year we have attempted (with variable success) to obtain a solubilized fraction of the brush border of the rat small intestine and retain the functional activity of the original tissue. Hopefully, techniques used to obtain this preparation can be scaled down and applied to intestinal biopsy specimens from the human small intestine.

Significance to Biomedical Research and the Program of NIAMD:

These cellular transport processes are of fundamental importance in all of mammalian metabolism, so that the overall significance of these studies is broader than the program of any one Institute.

The studies of cation transport may help clarify the basic mechanism by which these substances cross plasma membranes. They may also help to elucidate the pathogenetic defects in disease states (such as Bartter's syndrome, Liddle's syndrome and cystic fibrosis) where an alteration of membrane cation transport appears to be an integral part of the basic abnormality.

Successfully maintaining stable homogeneous lines of cultured brain cells provides the opportunity to investigate central nervous system physiology at the cellular and subcellular level. The specific physiologic functions of glial cells are currently unknown; however, electrophysiologic data suggest that they may regulate the composition of the extraneuronal environment and by so doing, modify electrical activity. Thus, these cells may play an intimate role in the pathogenesis of various seizure disorders. The marked dependence of astrocyte volume on the cationic composition of their external environment suggests a possible explanation of cerebral edema at the cellular level and perhaps, at the level of the plasma membrane. A high temperature coefficient for cation transport in both hamster and human astrocytes suggests a possible role of these cells in the genesis of the central nervous system symptoms in hypo- and hyperthermic states.

Characterization of amino acid transport in normal human tissues should further our understanding of the nature of the basic abnormality in various inborn errors of amino acid transport such as cystinuria and Hartnup's disease.

Being able to obtain functionally viable fractions of the plasma membrane in solubilized form affords one the opportunity of identifying the chemical composition of that portion of the membrane whose primary function is the translocation of substrate molecules.

Proposed Course:

These projects will be continued and the findings explored further.

Honors and Awards:

None.

Publications:

Gardner, J. D. and Lapey A.: Sodium outflux and ATPase activity in human and rabbit erythrocytes. J. Appl. Physiol. In press.

Gardner, Jerry D. and Ginzler, E. R.: Sodium transport in human erythrocytes--absence of an effect of prostaglandin E_1. Biochem. Biophys. Res. Comm. 42: 1063-1067, 1971.

Serial No. NIAMD-DHDB-5(c)
1. Digestive and Hereditary Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of diseases with altered membrane transport

Previous Serial Number: NIAMD-MDB-14(c), NIAMD-MDB-9(c)

Principal Investigator: Dr. Jerry D. Gardner

Other Investigators: Drs. Shlomo Shibolet, Artemis Simopoulis, Lynn Taussig, Arnold Levy and Leonard Laster

Cooperating Units: Metabolic Diseases Branch, NIAMD
Endocrinology Branch, NHLI
Pediatric Metabolism Branch, NIAMD

Man Years:

Total:	1.0
Professional:	0.5
Other:	0.5

Project Description:

Objectives:

1) To characterize the mechanisms by which the membrane transport of various substrates is altered in certain diseases; 2) to relate these alterations of membrane transport to the pathogenesis and clinical manifestations of the disease.

Methods Employed:

By using slices of intact tissue, isolated cells or isolated plasma membranes, and by using radioactive tracers, we studied the transmembrane movement of various substrates. Metabolism of the transported substrate was evaluated by use of techniques appropriate to the substrate being studied.

Patient Material:

Erythrocytes were obtained from 78 normal human subjects, 2 patients with Liddle's syndrome, 8 patients with Bartter's syndrome, 8 parents of patients with Bartter's syndrome, 9 siblings of patients with Bartter's syndrome, 9 patients with secondary hyperaldosteronism from other causes,

and 1 patient with primary hyperaldosteronism. Saliva was obtained from 16 normal human subjects and 12 patients with cystic fibrosis.

Major Findings:

1. Membrane transport in Bartter's syndrome and Liddle's syndrome (Drs. Jerry D. Gardner, Shlomo Shibolet, and Artemis P. Simopoulos)

Sodium influx and fractional sodium outflux, but not sodium concentration, are significantly increased in erythrocytes from patients with Liddle's syndrome. These alterations of sodium transport are not attributable to circulating levels of aldosterone, renin, angiotensin or serum potassium. The response of fractional sodium outflux and sodium influx to ouabain, ethacrynic acid and to changes in the cation composition of the incubation medium suggests that the increased sodium fluxes in Liddle's syndrome do not result solely from a quantitative increase in those components of sodium transport which occur in normal human erythrocytes. Instead, at least a portion of the increased erythrocyte sodium transport in Liddle's syndrome represents a component of sodium transport which does not occur in normal human erythrocytes.

In erythrocytes from 6 of 8 patients with Bartter's syndrome, fractional sodium outflux was decreased, erythrocyte sodium concentration was increased and sodium influx was normal. In erythrocytes from the other two patients with Bartter's syndrome fractional sodium outflux, erythrocyte sodium concentration and sodium influx were normal. First degree relatives of patients with abnormal sodium transport also tended to have altered erythrocyte sodium transport while first degree relatives of patients with normal erythrocyte sodium transport had normal erythrocyte sodium transport.

These findings indicate that there are at least two types of Bartter's syndrome and that the altered erythrocyte sodium transport present in one type is inherited.

Studies are in progress to evaluate cation transport in other tissues from patients with Liddle's syndrome and from patients with Bartter's syndrome.

2. Membrane transport in aminoacidurias (Drs. Jerry D. Gardner, Arnold Levy and Leonard Laster)

The transport of dibasic amino acids and cystine in erythrocytes from patients with cystinuria does not differ significantly from that in normal human erythrocytes. Studies are in progress to evaluate dibasic amino acid transport in other tissues from patients with cystinuria.

3. Membrane transport in cystic fibrosis (Drs. Jerry D. Gardner and Lynn Taussig)

Previously described abnormalities of exocrine cation transport in cystic fibrosis have been attributed to the presence of a circulating, uncharacterized substance which is also present in mixed saliva from patients with cystic fibrosis.

The present studies constitute attempts to develop a simple, reproducible bioassay system for the presence of this "sodium inhibitory factor." In contrast to two reports (both in abstract form) we detected no significant effect of fresh saliva from patients with cystic fibrosis on either sodium or alanine transport by rings, slices or sacs of rat small intestine in vitro.

Significance to Biomedical Research and the Program of NIAMD:

The studies of erythrocyte sodium transport in Bartter's syndrome and Liddle's syndrome indicate that both diseases may represent a primary, generalized, inherited abnormality of membrane cation transport. Studies of amino acid transport in tissues from patients with cystinuria offer the potential for characterizing the nature of the primary defect in this genetic disease. The studies of membrane transport in cystic fibrosis are directed toward finding a simple, reproducible technique for detection of the disease as well as for detecting the heterozygous carrier state.

Proposed Course:

These projects will be continued and the findings explored further.

Honors and Awards:

None

Publications:

Lapey, A. and Gardner, J. D.: Abnormal erythrocyte sodium transport in cystic fibrosis. Pediatric Research In press.

The major research programs of the Branch continued their emphasis on thyroid biochemistry, polypeptide hormones, control of cell processes and diabetes. There were no changes in permanent staff but Dr. Robert Perlman has accepted an appointment in the Department of Physiology, Harvard Medical School. Collaborative programs with the thyroid research groups at the University of Naples (G. Salvatore) and the University of Rome (M. Andreoli) have continued, and one has been initiated with the University of Marseilles (S. Lissitzky). The Branch was host to extended visits by Dr. Raymond Michel (College de France), Dr. S. Lissitzky (Univ. of Marseilles), Dr. Monte Greer (Univ. of Oregon) and Dr. A. Nishinaga (Univ. of Osaka). Dr. Harold Edelhoch served as Visiting Professor at Cornell University, Ithaca, New York and Dr. Jacob Robbins was a Visiting Professor at Gunma University, Maebashi, Japan. Guest workers from abroad included scientists from France, Great Britain, Hungary, Italy and Japan.

I. Thyroid Biochemistry

A. Thyroid Cell Membranes. Their Role in Iodide Transport, TSH Actior and Thyroid Secretion

Localized events in the plasma membrane of the thyroid cell are involved in thyroid hormone secretion. The process is initiated by binding of TSH to the basal membrane receptors which causes activation of adenyl cyclase and production of 3',5'-adenosine monophosphate (cAMP). This is followed by endocytosis of colloid at the apical cell membrane. The role of microtubules in the latter process was investigated. Colchicine and other microtubule-active agents blocked the stimulatory effect of TSH or dibutyryl cAMP on hormone release from mouse thyroid glands *in vitro* and prevented the formation of colloid droplets. Since adenyl cyclase and cAMP-phosphodiesterase were unaffected, these effects constitute evidence that the microtubules are involved in endocytosis. A soluble 6 S protein was shown to interact with [^{3}H]colchicine and is similar to the microtubule subunit found in other cells. Further studies on these structures as well as the microfilaments should elucidate the mechanism by which thyroglobulin stored in the colloid of the thyroid follicle is taken into the cell for proteolysis and hormone release (Wolff, Williams).

B. Thyroxine Synthesis and Iodination Reactions

In the model reaction for thyroxine (T_4) synthesis in thyroglobulin (TG) in which diiodohydroxyphenylpyruvic acid (DIHPPA) is reacted with the protein a considerable amount of 3,3',5'-triiodothyronine (T_3') is formed. Since this amino acid is not prevalent in native thyroglobulin, the reaction is a questionable model for *in vivo* hormone synthesis. Whereas DIHPPA addition to iodine-rich TG produced new iodothyronine residues with a T_4/T_3' molar ratio of 4:1, the use of iodine-poor TG gave a ratio of 1:3. This reflects the prevalence of monoiodotyrosyl (MIT) residues in iodine-poor TG.

To evaluate this model reaction further, virtually iodine-free human goiter TG was iodinated with 100 or 200 moles of I_2/mole TG. This resulted in the formation of approximately equal amounts of 3',3,5-triiodothyronine in addition to T_4 and moniodohistidine. The ratio of T_4/T_3 ranged from 3 to 6:1. On the other hand, rat TG iodinated *in vivo* to a similar extent (studied by equilibrium labeling) contained 3 to 4 times more T_3 and T_4, less monoiodohistidine, and some 3,3'-diiodothyronine. The ratio of T_4/T_3' was 15:1. Thus, neither reaction provides an exact model for *in vivo* hormone synthesis unless the results are influenced by species differences (Cahnmann, Ogawara).

C. Iodoproteins

In the course of studies aimed at elucidating the primary structure of thyroglobulin considerable complexity in the polypeptide chain structure has been encountered. Conditions for complete reduction of the S-S bands were perfected. When completely reduced and alkylated, and studied by polyacrylamide gel electrophoresis in SDS, bovine TG gave rise to 12 to 14 bands with molecular weights ranging from 10,000 to 330,000. All bands except the fastest contained carbohydrate. The heavier components could by dissociated by exposure to urea and guanidine, giving units with MW of 22,000 to 55,000 (Bilstad, Rall, Edelhoch, Salvatore).

In order to evaluate further the polypeptide chain structure of TG, studies on the closely related 27 S iodoprotein were performed. The structures of the 19 S and 27 S proteins differ since the latter could be dissociated without reduction to units having molecular weights of 30,000, 40,000 and 60-80,000. Under these conditions, the smallest unit arising from 19 S TG was MW 330,000. Since the amino acid composition and immunochemical properties of the 19 S and 27 S proteins are very similar, if not identical, it is likely that the difference in subunit structure represents different degrees of subunit cross-linking (Salvatore, Edelhoch, Bilstad and Rall).

Others have reported that freezing of TG in the presence of methylmercaptomidazole (MMI) affects TG structure. A study of this phenomenon with human and bovine TG indicated that MMI acts as a denaturant and liberates an 8 S species (MW 185,000) which ordinarily is not observed without prior reduction of disulfide bands. The iodine content of TG had no influence on the reaction (Schneider, Edelhoch).

The so-called "maturation" of TG was studied after ^{14}C-amino acid incorporation in guinea pig thyroid. A temperature-dependent, reversible interconversion between 19 S, an unfolded 15 S and a dissociated 12 S species occurred, with cold temperature favoring the latter two species. Poorly iodinated TG showed the same behavior. At 22°, TG was entirely in the 19 S form (Schneider, Edelhoch).

D. Thyroid Hormone Transport

Conditions for the purification of thyroxine-binding globulin (TBG) of human serum by affinity chromatography have been developed. Substitution of agarose with thyroxine or N-(6-aminocaproyl)thyroxine provided equally

potent adsorbants. The occurrence of non-specific protein adsorption prevented complete purification by this method, but a 1000-fold purification in 50% yield was obtained in a single column passage. Subsequent preparative electrophoresis on polyacrylamide gel gave a single component with thyroxine-binding activity (Pages, Cahnmann, Robbins).

Highly purified thyroxine-binding prealbumin (TBPA) of human serum was obtained from Dr. G. Schwick of Behringwerke, and was also prepared in the laboratory. Further purification gave a single component. The molecular weight (equilibrium sedimentation and polyacrylamide gel electrophoresis) was 54,000 and was reduced to 14,000 after exposure to 6 M guanidine. The tetramer was found to be remarkably stable, since it was not dissociated by 8 M urea, 4.5 M guanidine or pH 2.2. Furthermore, no change in molecular conformation (studied by fluorescence, polarization, UV-difference spectroscopy and circular dichroism) occurred between pH 3.5 and 12. CD studies revealed ∿50% β-structure and no α-helix. Below pH 3.5, a reversible structural change occurred in which there was exposure of aromatic residues to solvent but little molecular expansion. Fluorescence studies revealed competitive binding of 2 moles of 1-anilino-8-naphthalene sulfonate (ANS) and 1 mole of thyroxine. The binding affinity for thyroxine was reduced below pH 7.4. Above pH 7.4, the affinity constant for ANS was $\sim 10^{5}$ $1M^{-1}$, and for thyroxine $\sim 10^{8}$ $1M^{-1}$. Further studies on the binding reaction, together with X-ray diffraction studies now underway in Oxford, England, should soon provide a complete description of the T_4 binding site of this protein (Branch, Edelhoch and Robbins).

E. Mechanism of Thyroid Hormone Action

Recently published evidence suggests that control of $(Na^{+} + K^{+})$-ATPase activity may be one of the primary sites of action of thyroid hormone. Studies of the effects of thyroxine on the kinetic parameters of this enzyme have therefore been initiated since a hormonally induced change in the K_m for Na^{+}, or ATP (as opposed to a change in V_{max}) would be indicative of a direct effect of thyroxine on the enzyme. Thus far no effect on V_{max} of *in vitro* thyroid hormone administration in the range from 10^{-7} - 10^{-5} M has been observed on either the Mg^{++}-ATPase or the $(Na^{+} + K^{+})$ ATPase of euthyroid rat brain. Studies on hypothyroid animals and the effects of *in vivo* thyroxine administration are underway.

It has also been suggested that thyroid hormone may act primarily via modification of such cell membrane electrical properties. A brain slice system in which cyclic AMP levels are sensitive to a wide range of membrane depolarizing agents has been studied. Physiological concentrations of thyroid hormone markedly inhibited the stimulation of cyclic AMP levels caused by K^{+}, veratridine, and histamine. Stimulation due to ouabain was only slightly inhibited. Baseline levels of cyclic AMP remained unchanged in the presence of the hormone. Studies using direct electrical stimulation to increase cyclic AMP levels as well as an investigation on the effects of thyroid hormone on isolated enzymes which may affect the levels of cyclic AMP are planned (Gruenstein, Rall).

F. Studies on Thyroid Disease

Previous studies showed that Li^+, which occasionally produces goiter in psychiatric patients receiving lithium carbonate, causes a reduction in thyroid iodine secretion and blocks the effect of TSH. Although large doses depress iodine uptake and inhibit thyroid adenyl cyclase, the effect at ordinary therapeutic levels is mainly on the secretory process and probably occurs at a step between cAMP generation and colloid droplet formation.

This action of Li^+ might be useful in the therapy of Graves' disease. Only iodide is known to have a similar effect, and has the disadvantage of serving as substrate for hormone synthesis. Therefore the effect of lithium carbonate was studied on iodine kinetics in six patients with hyperthyroidism and diffuse goiter. In all subjects, Li^+ caused an acute decrease of 50% or greater in the rate of hormone secretion, and a resultant decrease in the circulating hormone level. Although escape from the Li^+ effect occurred due to continued accumulation of thyroid iodine, it is evident that Li^+ may be an effective therapy when rapid reduction of hormone secretion is required, such as in thyroid crisis. When combined with an agent such as methyl-mercaptoimidazole, which blocks thyroid iodine organification, the therapy is even more effective (Temple, Wolff, Robbins, Berman and Murphy).

II. Polypeptide Hormones

A. Structure

Previous studies on the structure of bovine growth hormone and ovine prolactin revealed a remarkable homology between these proteins with respect to molecular transitions occurring in urea and when exposed to pH changes. It has now been found that human chorionic somatomammotropin has closely similar properties as revealed by circular dichroism, fluorescence, absorption and polarization. Human growth hormone is similar but the resemblance is not so close as among the other three proteins. The structural homologies are borne out by similarities in amino acid sequence. It seems likely that these proteins are derived from a common ancestor by gene duplication (Aloj and Edelhoch).

B. Gonadotropins

Serum LH and testosterone were measured in 5 uremic subjects. LH was within normal limits despite the fact that testosterone levels were well below the normal range. Hemodialysis produced an increase in testosterone but no change in LH levels. The findings suggest a defect in LH release in uremia (Rosen and Weintraub).

C. Ectopic Chorionic Polypeptide Hormones

It was previously reported that circulating chorionic somatomammotropin (HCS) occurred in 11 of 128 patients with non-trophoblastic carcinoma. A prospective screening program in patients with bronchogenic carcinoma is now underway. In addition to 2 further cases having HCS, chorionic thyrotropin

was found in 1 patient. The circulating gonadotropin in 9 patients was identified as chorionic gonadotropin (HCG) by an antiserum which distinguished between HCG and LH. Chorionic alkaline phosphatase was also found in 4 patients, in 3 of whom it was associated with HCG or HCS. It is apparent that the secretion of chorionic proteins by bronchogenic tumors is discordant, suggesting non-linked synthetic mechanisms (Rosen, Weintraub and Sachson).

In connection with these studies the radioimmunoassay of HCS was significantly improved by the use of antibodies having greater affinity and specificity for the hormone. These were obtained by affinity chromatography of antisera on columns of HCS-coupled agarose (Weintraub and Rosen).

D. Insulin

To gain insight into the cause of diabetes and related diseases, new tools are being developed to study insulin secretion, insulin transport in plasma and insulin action.

The intracellular mechanisms of insulin secretion are under study, using islet cell tumors from hamsters as a model. Glucagon, a potent stimulator of insulin secretion in vivo, was found to bind to subcellular particles of the β-cell, as well as to activate adenyl cyclase and elevate the intracellular concentrations of cyclic 3'5'-AMP (Goldfine). The widely-used drug, tolbutamide, at physiological concentrations was found to inhibit competitively the phosphodiesterase of β-cells as well as in many other tissues accounting, in part, for the pancreatic and extrapancreatic effects of this drug, which is used widely in treating patients with diabetes (Goldfine and Roth).

In further studies of the nature of the circulating insulin in man, unique forms of proinsulin and insulin were found in the plasma of a man with an islet-cell carcinoma; these are the first such abnormal components described in man (Gorden).

The first step in the mechanism by which insulin acts on its target tissues was studied using specially prepared ^{125}I-insulin and purified membranes from liver. The specificity and quantitative characteristics of this interaction between insulin and its cellular binding sites have been studied in detail (Freychet and Roth).

E. Growth Hormone

Conventional irradiation and progestational agents are being studied in the treatment of acromegaly. The complications of acromegaly are under continuing study, especially the possible relationships between elevated growth hormone concentrations and cardiovascular diseases (Gorden and Roth).

F. Vasopressin and Oxytocin

In addition to continuation of the study of plasma vasopressin in normal and abnormal states of water balance, new studies have been undertaken to

develop tools for direct measurements of the action of these hormones on their target tissues (Thompson, Beardwell and Roth).

III. Mechanism of Action of Cyclic 3'5'-AMP in Bacteria

Although cyclic 3'5'-AMP is known to be an important and ubiquitous intracellular regulator of cell function, especially of hormone activation of target tissues, its mode of action is poorly understood. The earlier finding that cyclic 3'5'-AMP is an important regulator of enzyme synthesis in bacteria has been extended. Present studies give the most detailed understanding by far of cyclic 3'5'-AMP's role as an intracellular regulator. The previously described cyclic AMP binding protein has been isolated and characterized; the interaction of cyclic AMP, cyclic GMP, cyclic AMP-binding protein,and DNA has been studied in detail using simple systems of known composition; and selected mutants have been isolated that confirm the details of the proposed models (Perlman, Emmer and Straub).

IV. Radiation Dosimetry

In connection with work on the NIH radiation committee, an effort is underway to apply the MIRD approach to the solution of problems in clinical radiation dosimetry. This particularly involves gamma ray exposure. Availability of these methods of calculation will put such dosimetry on a more solid foundation (Lewallen).

Serial No. NIAMD/CEB 1c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Nonenzymic Synthesis of Thyroxine (T_4) and 3,5,3'-Triiodothyronine (T_3)

Previous Serial Number: CEB 1c

Principal Investigator: H. J. Cahnmann, Ph.D.

Other Investigators: H. Ogawara, Ph.D., A. Nishinaga, Ph.D.

Cooperating Units: None

Man Years

Total: 1 1/2
Professional: 1 1/4
Other: 1/4

Project Description:

When a solution of 4-hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA) is treated with oxygen, the keto acid is converted into its hydroperoxide (T_4 precursor). This hydroperoxide reacts with mono- and diiodotyrosine (MIT and DIT) residues in thyroglobulin (TG) to form 3,3',5'-triiodothyronine (T_3') and thyroxine (T_4) residues respectively.

Iodine-rich and iodine-poor TG containing 54 atoms I/mole and 6-10 atoms I/mole respectively were treated with labeled DIHPPA. The number of newly formed iodothyronine residues was determined after pronase digestion and chromatographic fractionation.

In iodine-rich TG two new iodothyronine residues/mole TG were formed with a T_4/T_3' molar ratio of 4:1 (cf. last year's report). In iodine-poor TG one new iodothyronine residue/mole TG was formed with a T_4/T_3' molar ratio of 1:3 which reflects the prevalence of MIT residues in iodine-poor TG. Although the coupling yield was lower in iodine-poor TG, the relative coupling yield (iodothyronine residues formed/iodotyrosine residues present) was higher.

While formation of iodothyronine residues by in vitro iodination (coupling between two iodothyronine residues) takes place only at relatvely high iodine levels, both iodine-rich and iodine-poor TG form iodothyronine residues efficiently in the coupling reaction with DIHPPA.

When virtually iodine-free human goiter TG was iodinated with 200 moles of I_2/mole TG, roughly equal amounts of T_3, T_3', and 3',5'-diiodothyronine were formed in addition to T_4. Native (equilibrium-labeled) rat TG differed in various respects from the *in vitro* iodinated goiter TG although both contained about the same amount of iodine. The native TG contained three to four times more T_3 and T_4 and considerably less monoiodohistidine. It also contained some 3,3'-diiodothyronine which was not detected in *in vitro* labeled TG. It was more extensively hydrolyzed by pronase.

In vitro iodination of TG carried out with rigorous exclusion of oxygen yielded the same amount of T_4 as aerobic iodination. This indicates that phenoxyl radicals are--in contrast to the aerobic coupling of iodotyrosine residues in the von Mutzenbecher reaction--not involved in the iodine-promoted coupling.

Honors and Awards: None

Publications:

1. Ogawara, H. and Cahnmann, H. J.: Nonenzymic synthesis of thyroxine in thyroglobulin by coupling with 4-hydroxy-3,5-diiodophenylpyruvate. Proceedings of the Sixth International Thyroid Conference, Vienna, June 1970, in press.

Serial No. NIAMD/CEB 2c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Affinity Chromatography of Thyroxine Binding Globulin (TBG)

Previous Serial Number: CEB 4c

Principal Investigator: H. J. Cahnmann, Ph.D.

Other Investigators: R. Pages, Ph.D. and J. Robbins, M.D.

Cooperating Units: None

Man Years

Total: 1 3/4
Professional: 1 1/2
Other: 1/4

Project Description:

It has been pointed out in last year's research report, that a high degree of purification of TBG is achieved when serum is passed at pH 9 through a column of agarose to which thyroxine (T_4) or of N-(6-aminocaproyl)-thyroxine (AC-T_4) is covalently bound. On raising the pH to 11.2 TBG is eluted. Although the bulk of the other serum proteins is removed by washing the column at pH 9, some of them are only released at pH 11.2 together with TBG.

A large number of variables has been investigated with the aim of eliminating or reducing the contamination of the eluted TBG with other proteins. Most experiments were carried out with Ac-T_4. Frequently Cohn fraction IV-4 was used instead of serum.

When the degree of substitution is reduced considerably (to 0.03 mg Ac-T_4/ml agarose), TBG is still held on the column at pH 9. Elution of the column at pH 5 did not reduce the amount of non-specifically adsorbed protein. Neither a salt gradient (0-1 M KCl) nor a pH gradient (9.2-11.3) resulted in a separation of the nonspecifically adsorbed proteins from TBG. When T_4 was eluted with T_4 (instead of alkali) only albumin was detected as a contaminant. The recovery of TBG was low, perhaps on account of the low solubility of T_4 at pH 9. Because of this and also because an elution with T_4 would require a subsequent separation of T_4 from TBG, all further experiments were done using elution at pH 11.2. Examination of the pH 11.2 elution profile showed that TBG is slightly retarded relative to other

proteins adsorbed at pH 9. When a AC-T_4 (not a T_4) column is used. At a low degree of substitution (<0.1 mg/ml) the non-specific adsorption of certain proteins is mainly due to agarose itself and not a result of the modification of agarose (activation and coupling with T_4 or AC-4).

It was possible to obtain a 1000-fold purification of TBG in a single passage through a AC-T_4 column with a recovery of ~50%.

Further purification by preparative polyacrylamide disc gel electrophoresis removed all non-specifically adsorbed proteins. The TBG obtained gave a single band in analytical disc gel electrophoresis.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 3c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Physical Chemistry of Proteins

Previous Serial Number: CEB 5c

Principal Investigator: H. Edelhoch, Ph.D.

Other Investigators: R. Lippoldt, A. Schneider, M.D., Ph.D., S. Aloj, M.D., M. Andreoli, M.D., L. Crammarossa, M.D., G. Salvatore, M.D., I. Covelli, M.D.

Cooperating Units: Istituto Patologia Medica, Univ. of Rome, Rome, Italy, Istituto di Patologia Generale, Univ. of Naples, Naples, Italy and Istituto di Patologia, Generale Universita, di Perugia, Perugia, Italy

Man Years

Total: 4 1/4
Professional: 3
Other: 1 1/4

Project Description:

The effect of methylmercaptoimidazole (MMI) on low and normal iodine thyroglobulin from human and bovine species during freezing has been investigated. MMI acts as a denaturant and liberates an 8 S species of M = 185,000. A molecule this small has not heretofore been observed without prior reduction of disulfide bonds. Contrary to reports in the literature, no difference was found between low and normal iodine thyroglobulin in their behavior towards MMI.

Human chorionic somatomammotropin has been characterized by circular dichroism, fluorescence, absorption and polarization of DNS-labeled protein. Its properties closely resemble those of bovine growth hormone and ovine prolactin. It is evident that its conformation is similar to the latter two hormones and all three hormones are derived from a common ancestor by gene duplication.

The properties of human growth hormone have also been characterized by similar procedures. Though the resemblance to the previously studied hormones is less, it is nevertheless likely, that the conformation of human growth hormone conforms to the others.

The subunit composition of 27S thyroglobulin has been studied by analytical and sucrose gradient centrifugation, and equilibrium centrifugation. The subunits of 27 S are completely different from those of 19 S and species of 30,000, 40,000 and 60-80,000 have been isolated. Since the amino acid composition and immunological properties of these two species are very similar, if not identical, the larger species present in 19 S must be composed of the smaller ones present in 27 S. It is likely that the difference in sizes represent different degrees of disulfide cross-linking.

The distribution of labeled iodoamino acids in iodinated 19 S thyroglobulin has been analyzed for by radiochromatography after pronase digestion. It is shown that this method of analysis gives the same results as the spectral method for thyroxine as well as for MIT and DIT.

When C^{14}-amino acids are incorporated into guinea pig thyroglobulin, the radioactivity sediments as a single 19 S peak at 22°. When the temperature is reduced to 2°, a 15 S species is observed which represents an unfolded form of the native molecule. The 15 S slowly dissociates to half-sized subunits (12S) when kept at 2°. These reactions are reversible by increasing the temperature to 22°. This effect of temperature explains what had previously been referred to as the "maturation" of thyroglobulin. It has also been shown that poorly iodinated guinea pig thyroglobulin shows the same behavior as newly synthesized molecules.

The heterogeneity of iodine in thyroglobulin has been evaluated by equilibrium isopycnic centrifugation in 34.5% RbCl. The composition versus per cent iodine has been obtained by analyzing the centrifugal patterns with the aid of the SAAM computer program (Berman, NIH).

Honors and Awards: None

Publications:

1. Bornet, H. and Edelhoch, H.: Polypeptide hormone interaction. I. Glucagon detergent interaction. J. Biol. Chem. 246: 1785-1792, 1971.

2. Schneider, A. B., Bornet, H. and Edelhoch, H.: The effects of low temperature on the conformation of thyroglobulin. J. Biol. Chem. 246: April, 1971.

3. Aloj, S. M. and Edelhoch, H.: Conformational similarity of ovine prolactin and bovine growth hormone. Proc. Natl. Acad. Sci. 66: 830-836, 1970.

4. Edelhoch, H. and Lippoldt, R. E.: The properties of bovine growth hormone. IV. Circular dichroism of native and iodinated molecules. J. Biol. Chem. 245: 4199-4203, 1970.

5. Malan, P. G. and Edelhoch, H.: Nitration of human serum albumin and bovine and human goiter thyroglobulins with tetranitromethane. Biochem. 9: 3205-3214, 1970.

Serial No. NIAMD/CEB 4c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Measurement of Iodocompounds in Biological Materials

Previous Serial Number: CEB 6c

Principal Investigator: C. G. Lewallen, M.D.

Other Investigators: Hans Hoch, Ph.D.

Cooperating Units: Veterans Administration Center, Martinsburg, West Virginia

Man Years

Total: 1
Professional: 1
Other: 0

Project Description:

Effort has been expended in two principal channels which are as follows:

1. In the capacity of chairman of the NIH Radiation Committee the investigator's efforts have been directed primarily along the following lines:

(a) Routine business of the Committee which primarily involves processing of new applications for clinical use of radioactive materials, annual renewals of existing applications, and evaluation of training and experience qualifications of applicants.

(b) A critical review has been undertaken and about 90% completed of all NIH authorizations for clinical use of radioactive materials with respect to validity of biological assumptions in the light of current data and in the light of currently available more rigorous approaches to evaluation of radiation exposure.

(c) An effort is underway to establish at NIH the application of the MIRD approach to the solution of problems in clinical radiation dosimetry, particularly where gamma ray exposure is involved.

(d) A rather extensive literature search has been made in an effort to compile bibliographic and metabolic kinetic data for labeled compounds being used in clinical research at NIH. Such data is necessary for computation of

patient radiation exposure for evaluation of proposals submitted by investigators.

2. In collaborative work with Dr. Hans Hoch, previous studies had suggested the presence in human sera of a dialyzable compound capable of binding thyroxine, as indicated by slowing of the diffusion rate of thyroxine across semipermeable membranes. For some time it appeared that this thyroxine binding factor could be demonstrated by its effect on thyroxine Rf on thin layer chromatography. This was suggested by studies employing ^{127}I thyroxine using the ceric arsenite and Prussian Blue reactions as localizing indicators. Subsequent chromatographic studies using ^{125}I thyroxine and localization by film autoradiography failed to show the effect. Currently an attempt is being made to demonstrate and roughly quantitate this factor by measuring its effect on the solubility of thyroxine. Results to date using columns of ^{127}I thyroxine suggest considerable interference from serum components, particularly uric acid. These studies will probably be extended to measurements of the effect of the factor on thyroxine electrophoretic mobility and diffusion in gels.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 5c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Role of Cyclic 3',5'-AMP in E. coli

Previous Serial Number: CEB 8c

Principal Investigator: R. Perlman, M.D., Ph.D.

Other Investigators: M. Emmer, M.D., M. Straub, M.D.

Cooperating Units: MB-NCI

Man Years

Total: 2 3/4
Professional: 2 3/4
Other: 0

Project Description:

Objectives: To determine the mechanisms by which cyclic adenosine 3',5'-monophosphate (cyclic AMP) regulates gene expression in bacteria.

Major Findings: Previous studies have shown that cyclic AMP and a specific protein, the cyclic AMP receptor protein (CRP) are required for the synthesis of a large number of inducible enzymes in E. coli. Cyclic AMP and CRP regulate β-galactosidase synthesis by stimulating the synthesis of lac operon messenger RNA (mRNA); this action requires an intact lac promoter locus. The gene for the synthesis of CRP has now been mapped on the E. coli chromosome. This gene, crp, maps very near the genes for resistance to streptomycin (str) and spectinomycin (spc). CRP has been purified to homogeneity. The purified protein has a molecular weight of 45,000, and is composed of two similar or identical subunits. CRP is a basic protein, having an isoelectric point of 9.2. Both CRP and cyclic AMP are required for the synthesis of lac mRNA and β-galactosidase in vitro. CRP is apparently the only protein which is specifically required for cyclic AMP action, since the nucleotide stimulates lac mRNA synthesis in a system containing only CRP, RNA polymerase, and a source of lac operon DNA. A new lac regulatory mutant has been isolated. This mutant is responsive to cyclic AMP, but, in the absence of cyclic AMP or CRP, makes much more β-galactosidase than do wild-type cells. Because this mutation appears to be a promoter mutation, we have called it a superpromoter (P^s). CRP binds to DNA in vitro. This binding is almost completely dependent upon cyclic AMP, and is inhibited by cyclic guanosine 3',5'-monophosphate, a competitive inhibitor of cyclic AMP action. It is not yet clear whether this binding is specific

for specific DNA sequences (promoter loci?), or whether it is non-specific with respect to DNA. However, the most plausible model for the action of cyclic AMP and CRP is that they bind to DNA, thereby rendering the DNA a better template for transcription by RNA polymerase.

Proposed Course: To determine more precisely the mechanism by which cyclic AMP and CRP stimulate lac transcription. Binding studies between CRP and DNA will be pursued, in order to elucidate the DNA specficity of this interaction, to determine the kinetic and thermodynamic parameters of this binding, and to assess any changes in DNA structure resulting from the formation of the CRP-DNA complex.

Honors and Awards: None

Publications:

1. Emmer, M., deCrombrugghe, B., Pastan, I. and Perlman, R.: Cyclic AMP receptor protein of E. coli: Its role in the synthesis of inducible enzymes. Proc. Nat. Acad. Sci. 66: 480-487, 1970.

2. Pastan, I. and Perlman, R.: Cyclic adenosine monophosphate in bacteria. Science 169: 339-344, 1970.

3. Varmus, H. E., Perlman, R. L. and Pastan, I.: Regulation of lac transcription in Escherichia coli by cyclic adenosine 3',5'-monophosphate. J. Biol. Chem. 245: 6366-6372, 1970.

4. Perlman, R., Chen, B., deCrombrugghe, B., Emmer, M., Gottesman, M., Varmus, H. and Pastan, I.: The regulation of lac operon transcription by cyclic adenosine 3',5'-monophosphate. Cold Spring Harbor Symposium on Quantitative Biology 35: 419-423, 1970.

5. Pastan, I. and Perlman, R.: Cyclic AMP in metabolism. Nature 229, 5-8, 1971.

6. deCrombrugghe, B., Chen, B., Gottesman, M., Pastan, I., Varmus, H., Emmer, M., and Perlman, R.: Regulation of lac mRNA synthesis in a soluble cell-free system. Nature 230: 37-40, 1971.

7. Varmus, H. E., Perlman, R. L., and Pastan, I.: Regulation of lac transcription in antibiotic-treated E. coli. Nature 230: 41-44, 1971.

Serial No. NIAMD/CEB 6c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Thyroid Iodoproteins

Previous Serial Number: CEB 10c

Principal Investigators: J. Bilstad, M.D., H. Edelhoch, Ph.D., G. Salvatore, M.D., J. E. Rall, Ph.D., M.D.

Other Investigator: R. Lippoldt

Cooperating Units: DIR-NIAMD

Man Years

Total: 3 1/4
Professional: 2 1/4
Other: 1

Project Description:

The goal of this project is to determine the number and size of the primary chains of the thyroid protein, thyroglobulin (MW 660,000, 19 S) and to determine whether these chains are identical or non-identical. Thyroglobulin extracted from beef thyroid gland was purified by ammonium sulfate precipitation and agarose gel filtration. Upon exposure to 6 M guanidine-HCl or the detergent, SDS, there is incomplete dissociation into half-sized units (12 S). Conditions for complete reduction and alkylation of the protein were studied including the effects of temperature, pH, duration of the reaction and the use of different alkylating agents (iodoacetic acid, iodoacetamide, and acrylonitrile). The results were evaluated by SDS polyacrylamide gel electrophoresis and amino acid analysis. The total number of half-cystines was determined by performic acid oxidation and amino acid analysis and was found to be 250.

The SDS gel electrophoresis pattern of completely reduced and alkylated thyroglobulin is reproducible but complex and consists of 12 to 14 bands with molecular weights ranging from 10,000 to 330,000. The same pattern was obtained in SDS plus 7 M urea. All bands with the exception of the fastest migrating band contained carbohydrate, as shown by staining with PAS. No evidence for enzymatic or chemical hydrolysis was found when the native thyroglobulin was exposed to SDS for varying periods of time at 25° and 37°.

Each of the 12 protein bands was isolated by gel electrophoresis and run again in SDS disc gels to determine if aggregation of the individual segments would take place. However, each band migrated predominantly as the starting species with only very small amounts of dimer formation. There was also a small amount of further dissociation of the heavier components into lighter fragments. After exposure to urea followed by guanidine the band corresponding to MW 330,000 dissociated into considerably smaller units; the MW determined by sedimentation equilibrium ranged from 22,000 to approximately 55,000. Preliminary recombination experiments of selected bands rerun in SDS disc gels gave no indication of aggregation. Amino acid analyses of the eluted bands are in progress.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 7c
1. Clinical Endocrinology
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanism of Thyroid Hormone Action

Previous Serial Number: None

Principal Investigator: E. Gruenstein, Ph.D., J. E. Rall, Ph.D., M.D.

Other Investigator: J. Daly, Ph.D.

Cooperating Units: LC-NIAMD

Man Years

Total: 2 1/2
Professional: 1 1/2
Other: 1

Project Description

(a) Recently published evidence suggests that control of $(Na^+ + K^+)$-ATPase activity may be one of the primary sites of action of thyroid hormone. Studies of the effects of thyroxine on the kinetic parameters of this enzyme have therefore been initiated since a hormonally induced change in the K_m for Na^+, K^+, or ATP (as opposed to a change in V_{max}) would be indicative of a direct effect of thyroxine on the enzyme rather than an indirect effect via new protein synthesis. Thus far no effect on V_{max} of *in vitro* thyroid hormone administration in the range from 10^{-7} - 10^{-5} M has been observed on either the Mg^{++}-ATPase or the $(Na^+ + K^+)$ ATPase of euthyroid rat brain. Studies on hypothyroid animals and the effects of *in vivo* thyroxine administration on ATPase kinetics are underway.

(b) It has also been suggested that thyroid hormone may act primarily via modification of such cell membrane electrical properties as resistance or capacitance. In order to investigate this possibility a brain slice system whose cyclic AMP levels are sensitive to a wide range of membrane depolarizing agents, including K^+, veratridine, histamine, and ouabain, has been employed. Physiological concentrations of thyroid hormone have been found to markedly inhibit the stimulation of cyclic AMP levels caused by K^+, veratridine, and histamine. Stimulation due to ouabain was however only slightly inhibited by thyroid hormone. Baseline levels of cyclic AMP remain unchanged in the presence of the hormone. Studies using direct electrical stimulation to increase cyclic AMP levels as well as an investigation on the effects of thyroid hormone on isolated enzymes which may affect the levels of cyclic AMP are planned.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 8c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Protein Synthesis in the Thyroid Gland

Previous Serial Number: CEB 11c

Principal Investigator: Jacob Robbins, M.D.

Other Investigator: F. Monaco, M.D.

Cooperating Units: None

Man Years

Total: 1 1/2
Professional: 1 1/4
Other: 1/4

Project Description:

A new series of experiments employing the transplantable rat thyroid tumor line $1\text{-}1_c$, has been started to elucidate the defective release of thyroglobulin from subcellular particles described previously. Unfortunately the tumor had changed during serial transplantation. It now contains fewer mature follicles, accumulates only about 1% of administered radioiodine, and contains very little soluble iodoprotein. Nevertheless, most of this particulate iodoprotein could be solubilized with vigorous homogenation and digitonin, both procedures yielding soluble 19 S protein. The protein appears, therefore, to be a relatively complete thyroglobulin molecule. The next step will be to examine the final stage in thyroglobulin synthesis--the formation of polysaccharide chains.

Honors and Awards: None

Publications:

1. Salabe, G. B. and Robbins, J. The nature of particulate thyroid proteins in an experimental rat thyroid tumor. Biochim. Biophys. Acta 214: 198-206, 1970.

Serial No. NIAMD/CEB 9c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Thyroxine - Protein Interactions

Previous Serial Number: CEB 12c

Principal Investigators: W. Branch, M.D., H. Edelhoch, Ph.D. and J. Robbins M.D.

Other Investigators: J. E. Rall, Ph.D., M.D., M. V. Brackbill and G. Sandeen

Cooperating Units: DIR-NIAMD

Man Years

Total: 3 1/2
Professional: 1 1/4
Other: 2 1/4

Project Description:

Relationships between thyroxine binding and conformation were studied in a purified commercial preparation of human thyroxine binding prealbumin (TBPA). Molecular weight of 53,500 by equilibrium sedimentation in water compared to 14,000 in 6 M guanidine suggested four subunits of identical size. Studies with ultraviolet circular dichroism and by fluorescence quenching of tryptophan revealed one thyroxine binding site with $K_a \sim 10^8$ LM^{-1}. Affinity and number of sites were apparently unchanged between pH 10 and 7.4, whereas binding decreased significantly beyond these limits. 1-anilino-8-naphthalenesulfonate (ANS) bound to TBPA with increased fluorescence quantum yield and with a 44 nm shift of fluorescence emission maximum toward the ultraviolet, characteristics which indicate nonpolarity of its binding site. From ANS titration curves, two binding sites with $K_a \sim 10^5$ LM^{-1} were calculated. Occupancy of the single thyroxine site displaced ANS competitively from both sites, thereby furnishing independent measurements of thyroxine affinity. Protein structure was studied by ultraviolet difference spectroscopy, intrinsic protein fluorescence, circular dichroism and polarization of 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS) fluorescence. No change in molecular conformation could be detected over the pH range 3.5 to 12. Subunit structure remained intact in 8 M urea or 4.5 M guanidine at neutral pH and at pH 2.2 in aqueous solution.

Our studies are compatible with a tetrameric structure of remarkable

stability and with a single hydrophobic crevice for thyroxine binding. Changes in binding affinity do not appear to depend on conformational changes in the protein. Circular dichroism studies revealed almost no α-helical structure and about 50% ß-structure.

Purification procedures for rhesus monkey TBPA employing DEAE sephadex and polyacrylamide gel electrophoresis have been perfected and are being used to prepare quantities of the two genetic types sufficient for physical and chemical studies.

Honors and Awards: None

Publications:

1. Rall, J. E. and Robbins, J.: Transport des hormones thyroidiennes. In Doin and Masson et C^{ie} (Ed.): Metabolisme Peripherique et Transport Humoral des Hormones Thyroidiennes et Steroides. Paris, France, des Annales D'Endocrinologie, 1969, pp. 1-8.

Serial No. NIAMD/CEB 10c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Action of Thyroid Hormones

Previous Serial Number: None

Principal Investigators: H. Carlson, M.D. and J. Robbins, M.D.

Other Investigators: None

Cooperating Units: None

Man Years

Total: 1 1/2
Professional: 1 1/4
Other: 1/4

Project Description:

The possible role of the thyroid hormones on the function of ciliary epithelium is under exploration. Methods for studying the transport of particles on the mucosal surface of the explanted frog esophogus and mammalian trachea have been standardized. Optical methods for measuring the rate of the ciliary beat are under development. No response to thyroid hormones added in vitro has yet been observed.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 11c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Thyroid Disease

Previous Serial Number: CEB 13c

Principal Investigators: J. Robbins, M.D., R. Temple, M.D. and J. Wolff, Ph.D., M.D.

Other Investigators: M. Berman, Ph.D. and D. Murphy, M.D.

Cooperating Units: Mathematics Research, NIAMD; Clinical and Behavioral Research, NIMH

Man Years

Total: 1 3/4
Professional: 1 1/2
Other: 1/4

Project Description:

Lithium ion blocks the release of iodine from the rat thyroid in a manner similar to iodides. Since iodides obscure diagnostic thyroid function tests and serve as a substrate for hormone synthesis, we investigated Li^+ as an alternative therapeutic agent when a rapid fall in hormone level is desired.

Four hyperthyroid patients were studied during a control period, a 2 to 3 week period of lithium carbonate therapy (serum Li^+ = 0.5 to 1.2 meq/L) and a second control period. Conventional parameters of thyroid function were measured and the distribution of a tracer dose of ^{131}I into the thyroid, serum and urine was followed.

In every patient, Li^+ treatment produced a prompt decrease in the rate of ^{131}I loss from the thyroid gland, a fall in serum ^{131}I-PBI and urinary ^{131}I excretion, and a fall in serum T_4 associated with decreased clinical evidence of hyperthyroidism. The mean fall in serum T_4, achieved within six days, was 28%. Multi-compartment analysis revealed that the principal kinetic parameter affected was the fractional hormone secretion rate, which was reduced by ~50%. We conclude that Li^+ at non-toxic serum levels, can promptly lower circulating thyroid hormone concentrations in thyrotoxic patients by blocking hormone release. Since the thyroidal iodide clearance was not decreased, the calculated glandular iodine content was increased, corresponding to the measured increase in glandular iodine found in rats.

Thus, even with persisting inhibition of the fractional hormone release rate, the absolute amount of hormone released will gradually rise. This is, in fact, observed. Hence, Li^+ alone is beneficial principally for short-term treatment of hyperthyroidism: since Li^+ is effective in the presence of mercaptoimidazole, combined therapy appears to be promising.

Lithium will probably be useful in the therapy of thyrotoxic crisis and for rapid control of the hyperthyroid state.

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 12c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Gonadotropin Studies

Previous Serial Number: CEB 14c

Principal Investigator: S. W. Rosen, Ph.D., M.D.

Other Investigators: B. D. Weintraub, M.D., R. Sachson, M.D. and I. Stotler, B.S.

Cooperating Units: NCI Oncology Unit, Veterans Administration Hospital, Washington, D. C., Renal Division, Peter Bent Brigham Hospital, Boston, Mass., Pathology Dept., Stanford Univ. Medical Center, Palo Alto, Calif., Reproduction Research Branch, NICHD

Man Years

Total: 4 1/4
Professional: 3
Other: 1 1/4

Project Description:

I. Ectopic Polypeptide Production by Tumors

Studies continue on the production of polypeptides by tumors, particularly those characteristic of the placenta and expressed in pregnancy but highly repressed and not detected (by current methods) in the nonpregnant adult. Such polypeptides serve as markers for the presence of neoplasm.

We are in the process of prospectively screening for several such markers all cases of bronchogenic carcinoma admitted to the DC VA Hospital Oncology Unit.

A. Ectopic Chorionic Gonadotropin (HCG)

A total of 9 patients with increased urinary gonadotropin (bioassay), increased serum concentrations of gonadotropin (immunoassay) and non-trophoblastic cancers have been studied. In all of these cases that have been examined, the serum gonadotropin was not distinguished from chorionic gonadotropin (HCG), but different from LH, in gel chromatography and in radioimmunoassay using selected anti-HCG and anti-LH antisera that discriminated between these two gonadotropins.

In collaboration with Dr. Howard Sussman of Stanford, we are trying to design rigorous immunofluorescent and immunohistochemical experiments that will permit us to determine the precise cells in these tumors (which are often heterogeneous) that are making the HCG.

B. Ectopic Chorionic Somatomammotropin (HCS)

We have previously detected the ectopic production of HCS in 11 of 128 patients with various tissue-proved malignancies other than those originating in trophoblast. Currently we are designing a prospective study to determine the incidence of HCS production by bronchogenic carcinomas. In a preliminary survey we have already found two additional cases.

C. Ectopic Chorionic Thyrotropin (HCT)

Using a still insensitive (heterologous) radioimmunoassay, Sachson has obtained data suggestive of ectopic HCT production in a man with bronchogenic carcinoma (one of the cases in the D.C. VA Hospital screening study). Serum levels of this putative HCT fell after radiotherapy directed against the patient's neoplasm.

D. Ectopic Placental Alkaline Phosphatase (PAP)

Using an antiserum that distinguishes PAP from other organ-specific isoenzymes, Sussman at Stanford found PAP in the serum of 4/5 of our cases with non-trophoblastic tumors that he examined. Of these four, three had serum HCG or HCS whereas in one case PAP was not associated with either HCG or HCS (<1 ng/ml). The other case of nontrophoblastic tumor had undetected PAP but very high serum HCG and HCS.

These data showing discordant neoplastic production of 3 placental proteins suggest non-linked synthetic mechanisms and contrast with the concordance of neoplastic ACTH-βMSH production.

II. Miscellaneous Endocrine Studies

A. Serum Testosterone and Gonadotropins in Uremic Men

In five untreated uremics studied in collaboration with Dr. George Bailey of the Peter Bent Brigham Hospital, Boston, serum testosterone was low (mean 0.19 µg/100 ml; normal 0.3-1.0) but serum LH was within normal limits or only slightly elevated. Sera after hemodialysis revealed a rise in testosterone to a mean of 0.45; LH remained within normal limits.

These data agree well with those of Wieland *et al.* and suggest a defect in central LH release in uremia.

B. Characterization of HCS

Weintraub has been studying the physicochemical and immunologic characteristics of HCS from normal placenta as well as from malignancies of trophoblastic and nontrophoblastic origin. In addition, we are studying the nature of HCS synthesized in vitro by means of ^{14}C-amino acid incorporation with tumor explants and various clonal lines in tissue culture. The latter studies (in collaboration with Drs. Peter Kohler and William Bridson of NICHD) have already shown HCS production by various clones in amounts not correlated with HCG production.

C. Affinity Chromatography

Weintraub has developed methods of concentrating and purifying HCS and other hormones by means of affinity chromatography. Anti-HCS has been coupled to agarose (Sepharose) by means of cyanogen bromide activation; the substituted matrix specifically binds HCS, which can be eluted by 3-6 M guanidine. Recently we have coupled HCS to Sepharose and used this to fractionate anti-HCS by means of elution with a guanidine gradient. The resulting antibody fractions were relatively homogeneous and differed widely in affinity and specificity for HCS. Certain antibody fractions have already proved of great benefit in augmenting the sensitivity of the radioimmunoassay.

Honors and Awards: None

Publications:

1. Weintraub, B. D. and Rosen, S. W. Ectopic production of human chorionic somatomammotropin by non-trophoblastic cancers. J. Clin. Endocrinol. 32: 94-101, 1971.

Serial No. NIAMD/CEB 13c
1. Clinical Endocrinology Branch
2. Diabetes & Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Interaction of Polypeptide Hormones with Their Receptors in Target Tissues

Previous Serial Number: CEB 15c

Principal Investigators: P. Freychet, M.D., C. R. Kahn, M.D., A. Sober, M.D., J. R. Gavin, Ph.D. and J. Roth, M.D.

Other Investigator: D. M. Neville, Jr., M.D.

Cooperating Units: NIMH-LNC

Man Years:

Total: 3 3/4
Professional: 3 1/2
Other: 1/4

Project Description:

The first step in insulin action is binding to specific receptors on the plasma membrane of target cells. For direct study of this step, iodoinsulin was prepared by iodinating at a low iodide to insulin ratio and separating iodoinsulin from uniodinated insulin by chromatography on DEAE-cellulose. Pure ^{127}I-monoiodoinsulin, whose composition was verified by spectral titration, retained full bioactivity (25 U/mg) measured as stimulation of glucose oxidation in isolated fat cells.

^{125}I-insulin of high specific radioactivity prepared by this method bound to plasma membranes purified from rat liver; with ^{125}I-insulin at 5×10^{-10} M 10-50% of the radioactivity was bound. Twenty percent of the bound ^{125}I-insulin was displaced by unlabeled insulin at 6 ng/ml (10^{-9} M), equivalent to the insulin concentrations in portal blood. Displacement was 80% with 10^{-7} M and 90% with 10^{-5} M insulin. Insulins with biological potencies that differed over a 100-fold range: pork, human, beef, fish and guinea pig insulin, porcine proinsulin, desalanine-desasparagine and desoctapeptide-insulin, displaced ^{125}I-insulin from liver receptors in proportion to their ability to stimulate glucose oxidation in fat cells. Insulin chains, inactive in fat cells, produced no displacement of labeled insulin. Glucagon, ACTH and HGH were also without effect. Binding was more rapid and more complete at 30° than at 0°. Addition of excess insulin produced rapid dissociation of ^{125}I-insulin. The amount of insulin bound per mg of liver protein increased 50-fold as plasma membrane was purified from crude

homogenate. Treatment of purified plasma membrane with trypsin destroyed its ability to bind insulin.

For the interaction of insulin with its receptor at 30°, K, the apparent equilibrium constant = 6 10^8 and k_1/k_2, the apparent association/dissociation constants = 8 x 10^8. We estimate that each liver cell has approximately 150,000 of these sites per cell.

Honors and Awards: None

Publications:

1. Lefkowitz, R., Roth, J. and Pastan, I.: Radioreceptor assay of adrenocorticotropic hormone: New approach to assay of polypeptide hormones in plasma. Science 170: 633-635, 1970.

2. Lefkowitz, R. J., Roth, J. and Pastan, I.: Effects of calcium on ACTH stimulation of the adrenal: Separation of hormone binding from adenyl cyclase activation. Nature 228: 864-866, 1970.

3. Freychet, P., Gorden, P. and Roth, J.: Monoiodoinsulin: Demonstration of its biological activity and binding to fat cells and liver membranes. Biochem. Biophys. Res. Commun. In press.

4. Freychet, P., Roth, J. and Neville, D. M.: Insulin receptors in the liver: Specific binding of ^{125}I-insulin to the plasma membrane and its relation to insulin bioactivity. Proc. Nat. Acad. Sci. In press.

Serial No. NIAMD/CEB 14c
1. Clinical Endocrinology Branch
2. Diabetes & Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Intracellular Mechanisms of Insulin Synthesis and Release

Previous Serial Number: none

Principal Investigators: Ira Goldfine, M.D., R. Perlman, M.D., J. Roth, M.D.

Other Investigators: none

Cooperating Units: none

Man years:

Total: 1 3/4
Professional: 1 1/2
Other: 1/4

Project Description:

Glucagon stimulates the beta cell to release insulin both *in vitro* and *in vivo*. We studied subcellular fractions of the insulin secreting islet cell tumor of the Syrian (golden) hamster. Tumors were homogenized in 0.001 M $NaHCO_3$ and centrifuged. The 10,000 xg particles contained adenyl cyclase activity (measured as conversion of $AT^{32}P$ to cyclic $AM^{32}P$) that was stimulated three-fold by glucagon. Other polypeptide hormones, isoproterenol, glucose, arginine and tolbutamide were without effect. Enzyme activity was linear for up to 20 minutes of incubation and proportional to protein concentration. After lyophilization the particles were stable at -20° for several months. ^{125}I-glucagon of high specific activity rapidly bound to the particles at 30°, reaching equilibrium in 15 minutes. With unlabelled glucagon at 0.1 ng/ml, 15% of the ^{125}I-glucagon was displaced; with unlabelled hormone at 100 ng/ml, 75% of labelled hormone was displaced. Secretin, pentagastrin and other polypeptide hormones were without effect. This system permits direct study of the interaction of glucagon with its binding site as well as receptor-cyclase interactions, and also provides the basis for a radioreceptor assay of glucagon.

Sulfonylureas, like tolbutamide, have both pancreatic (insulin secreting) and extrapancreatic effect but the intracellular site of action of these compounds is unknown. In insulin-secreting islet cell tumors of Golden hamsters, cyclic 3',5'-AMP phosphodiesterase was found in particulate and soluble fractions. Tolbutamide and other sulfonylureas acted as competitive inhibitors of phosphodiesterase with a K_i for tolbutamide of 5×10^{-3} M. The minimum inhibitory concentration of tolbutamide was 0.25×10^{-3} M which

approximates effective plasma concentrations in man. The two components of tolbutamide, butylurea and p-toluene sulfonic acid, had little effect even when present in combination. The inhibition of phosphodiesterase by tolbutamide was also found with purified beef-heart phosphodiesterase and with phosphodiesterase activity from rat lung, liver, kidney and brain.

Publications:

1. Emmer, M., Gorden, P. and Roth, J.: Diabetes in association with other endocrine disorders. Medical Clinics of North America. in press

Serial No. NIAMD/CEB 15c
1. Clinical Endocrinology Branch
2. Diabetes & Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Assays of Vasopressin

Previous Serial Number: CEB 16c

Principal Investigators: E. Thompson, M.D., C. Beardwell, M.D., J. Roth, M.D.
P. Gorden, M.D.

Other Investigators: M. Lesniak

Cooperating Units: None

Man Years

Total: 3 1/2
Professional: 2 1/4
Other: 1 1/4

Project Description:

To study directly the interaction of oxytocin with its biologically significant receptors, a hormone of high specific radioactivity is needed that retains biological activity. Oxytocin was iodinated at low ratio (1:10) of iodide to hormone and freed of unlabelled oxytocin by two passages over G-25 Sephadex; oxytocin-I, since it adsorbs loosely to the gel, is eluted after oxytocin. The product was shown to be pure monoiodooxytocin by spectral titration at 295, 305 and 325 mμ at pH 5 through 12.

In isolated fat cells, both oxytocin-I and oxytocin stimulated glucose oxidation. At 10^{-7} M, both hormones achieved their maximum effect. A 50-fold increase in concentration of either hormone produced no further stimulation. Since both dose response curves plateaued, and oxytocin-I at very high concentrations produced only 70% of the effect of oxytocin, it is clear that no significant part of oxytocin-I bioactivity was due to the presence of uniodinated oxytocin. Insulin in the same experiments produced a 3-fold greater effect. In homogenates of toad bladder epithelium, oxytocin and oxytocin-I stimulated the conversion of ATP to cyclic 3',5'-AMP. Both produced their maximum effect at 10^{-5} M. The extent of deiodination during incubation was negigible.

These results show that the introduction of one iodine atom into a polypeptide as small as oxytocin is compatible with the persistence of substantial bioactivity, extending the range of bioreceptor studies to small polypeptide hormones.

Honors and Awards: none

Publications: none

Serial No. NIAMD/CEB 16c
1. Clinical Endocrinology Branch
2. Diabetes & Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of Human Growth Hormone (HGH) in Man

Previous Serial Number: CEB 17c

Principal Investigators: P. Gorden, M.D., J. Roth, M.D.

Other Investigators: C. Hendricks

Cooperating Units: None

Man Years

Total: 1 1/4
Professional: 1/2
Other: 3/4

Project Description:

We have reported that in untreated acromegaly plasma GH concentration increases with time and that external irradiation from a conventional supervoltage source is an effective way to reduce GH concentration.

Thirty previously untreated acromegalic patients have been treated with 4000-4500 r supervoltage irradiation and followed for 1-6 years. All 30 patients were restudied at the end of 1-2 years and 16/30 were studied again at the end of 3-6 years.

Prior to treatment mean GH for 30 patients was 58 ng/ml, median 29 and range 7-220 ng/ml. By 1-2 years after irradiation there was a 65% reduction in plasma GH for the 30 patients (mean GH = 22 ng/ml, median 14 and range 3-85 ng/ml). Further reduction in plasma GH occurred with time and by 3-6 years post irradiation there was a 78% reduction in plasma GH for 16 patients (mean GH = 13 ng/ml., median 7 and range 3-50 ng/ml). In these 16 patients GH was less than 10 ng/ml in 2/3 of the group and less than 5 ng/ml in 1/3 of the group.

Three additional patients treated by transfrontal hypophysectomy had a 71, 49 and 10% reduction in GH and when subsequently treated by pituitary irradiation had a further 70, 50 and 47% reduction in GH respectively.

There are wide variations in benefit among individual patients with all modalities of therapy; the long term results of supervoltage irradiation is comparable to the reported results of other forms of irradiation or surgical therapy and is accompanied by trivial morbidity and no mortality.

Honors and Awards: None

Publications:

1. Roth, J. and Gorden, P.: Treatment of acromegaly. In Conn, H. (Ed.): Current Therapy (ed. 23), Saunders, 1971 pp. 386-388.

Serial No. NIAMD/CEB 17c
1. Clinical Endocrinology Branch
2. Diabetes & Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Nature of Plasma Insulin in Man

Previous Serial Number: 18c

Principal Investigators: P. Gorden, M.D. and J. Roth

Other Investigators: C. Hendricks

Cooperating Units: None

Man Years

Total: 1 1/4
Professional: 1/2
Other: 3/4

Project Description:

Big insulin (proinsulin-like component) represents a high proportion of circulating insulin in patients with islet cell tumors (26-80% of total basal insulin). We studied a patient with islet cell carcinoma in whom 80% of circulating insulin is a heretofore undescribed form (tumor big insulin). When plasma from this patient was filtered on G50 Sephadex, most of the plasma insulin immunoreactivity was recovered as a discrete peak ahead of both non-tumor big insulin and ^{125}I-proinsulin. In all previous studies of plasma from tumor and non-tumor subjects by us and others, all insulin immunoreactivity is recovered with proinsulin or beyond. Using an antiserum that does not distinguish between dilutions of porcine insulin, proinsulin, or non-tumor human big and little insulin, the tumor big insulin had a markedly different reactivity over a 100-fold range of dilutions. Tumor big insulin purified from plasma had 5.5 μU of bioactivity (glucose oxidation in isolated fat cells) per ng of immunoreactivity which is 3-fold greater than that of porcine proinsulin or non-tumor big insulin. Tumor big insulin was converted normally by exposure to trypsin. Release of tumor big insulin was not inhibited by large doses of diazoxide.

Little insulin from this patient's plasma had normal mobility on G-25 and was as active as porcine insulin in fat cells, but had the abnormal immunoreactivity found for the patient's big insulin.

It is likely that the proinsulin produced by this tumor is larger in size than heretofore described proinsulin-like substances and has one or more amino acid substitutions in the insulin part of the molecule.

This is the first demonstration in a human of a distinctive insulin.

Honors and Awards: None

Publication:

1. Sherman, B. M., Gorden, P., Roth, J. and Freychet, P.: Circulating insulin: The proinsulin-like properties of big insulin in patients without islet cell tumors. J. Clin. Invest. 50: 849-858, 1971.

Serial No. NIAMD/CEB 18c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Thyroid Plasma Membranes

Previous Serial Number: None

Principal Investigator: J. Wolff, M.D., Ph.D.

Other Investigator: A. B. Jones

Cooperating Units: None

Man Years

Total: 1 1/2
Professional: 1/4
Other: 1 1/4

Project Description:

Beef thyroid membranes have been purified about 100-fold on the basis of thyrotropin- or F^--stimulated adenyl cyclase activity. These activities co-purified with Na^+-K^+-requiring adenosine triphosphatase, K^+-stimulated p-nitrophenyl phosphatase, and 5'-nucleotidase.

The adenyl cyclase of these preparations is stimulated over a wide range of bovine thyrotropin concentrations, by human chorionic thyrotropin and, sometimes by prostaglandin E_1 but not by long-acting thyroid stimulator.

The adenyl cyclase is stable in the frozen state but loses thyrotropin and F^- responsiveness rapidly at 43°C. Maximal activity is obtained with a Mg^{++}/ATP ratio of 2 and when the ATP concentration exceeds 2 mM.

The thyrotropin response is enhanced by K^+ ion whereas the F^- response is not affected. Li^+ ion inhibits strongly, especially when the Mg^{++} concentration is limiting. Na^+ is weakly inhibitory.

Both F^- and thyrotropin-stimulated cyclcase activity are strongly inhibited by N-ethylmaleimide, p-chlormercuribenzoate, sodium dodecyl sulfate, filipin, sodium tetraphenylboron, valinomycin and monensin A. Another class of agents, which includes the phenothiazines, thymol, cobramine B and gramicidin S, inhibits thyrotropin stimulation of the enzyme but enhances the F^- response.

It is concluded that the thyrotropin "receptor" and the adenyl cyclase

(F^--activation) are distinct entities, and it is suggested that prostaglandin E_1 uses a different "receptor" than thyrotropin.

Honors and Awards: None

Publications:

1. Macchia, V. and Wolff, J.: The effect of a purified preparation of lecithinase c on iodide transport. FEBS Letters 10: 219-221, 1970.

2. Wolff, J.: La relation entre le transport et les effets inhibiteurs de l'iodure. Actual. Endocrinol. 11: 7-14, 1970.

Serial No. NIAMD/CEB 19c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Iodination of Histidine in Proteins

Previous Serial Number: CEB 19c

Principal Investigators: J. Wolff, M.D., Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years

Total: 0
Professional: 0
Other: 0

Project Description:

Terminated

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 20c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Thyroidal Iodide Transport

Project Serial Number: CEB 20c

Principal Investigator: J. Wolff, M.D., Ph.D.

Other Investigators: J. A. Williams, M.D., Ph.D., A. B. Jones

Cooperating Units: None

Man Years

Total: 0
Professional: 0
Other: 0

Project Description:

Inactive

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 21c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Role of Thyroid Hormones in Mitochondrial RNA Synthesis

Previous Serial Number: CEB 21c

Principal Investigators: J. Wolff, M.D., Ph.D. and J. E. Rall, M.D., Ph.D.

Other Investigators: None

Cooperating Units: DIR-NIAMD

Man Years

Total: 0
Professional: 0
Other: 0

Project Description:

Inactive

Honors and Awards: None

Publications: None

Serial No. NIAMD/CEB 22c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Effect of Lithium on Iodine Metabolism

Previous Serial Number: CEB 22c

Principal Investigators: J. Wolff, M.D., Ph.D., J. A. Williams, M.D., Ph.D.

Other Investigator:

Cooperating Units: NIHM (Dr. Dennis Murphy)

Man Years

Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

Lithium has been reported to be goitrogenic when used for the treatment of manic-depressive psychosis. To investigate the effects of lithium on iodine metabolism, male Sprague-Dawley rats were placed on a low iodine (LID) or normal iodine diet (NID) contained enough Li_2CO_3 to give serum lithium levels of 0.23-0.86 mEq/liter (human therapeutic range is 0.6-1.6 mEq/liter). The following effects were noted with lithium treatment: (a) thyroid weight increased concomitant with a slowing of thyroidal iodine release; (b) the ability to concentrate iodide was increased only after goiters were established; (c) on the LID, ^{131}I uptake was elevated throughout all phases of treatment, even when the release rate was normal; (d) iodine organification was unaffected but the proportion of ^{131}I present as iodothyronines was decreased; (e) the thyroidal ^{127}I content was increased; (f) despite these changes, the serum PBI remained normal as did the thyroxine turnover rate; and (g) thyrotropin (TSH) levels in serum were the same as controls except for a slight elevation early in the course of treatment; TSH levels did not correlate with goitrogenesis.

When LiCl was injected in large doses into intact rats (giving serum lithium levels of 3.08-3.89 mEq/liter), the iodide concentrating mechanism, ^{131}I uptake, and ^{131}I release rates were depressed. Similar experiments in hypophysectomized rats receiving TSH demonstrated these to be local antithyroid effects not mediated through the pituitary.

In rats, Li^+ is concentrated by thyroid tissue to a level 2.5-3 times greater than serum. This tissue/serum concentration ratio is attained within one hour following i.v. LiCl and is maintained in animals chronically treated with Li_2CO_3 for 153 days.

Lithium inhibits the TSH-induced stimulation of adenyl cyclase activity in beef thyroid membranes without affecting basal activity. Half-inhibition occurs at 4-8 mM Li^+ when the Mg^{++} concentration is 2.5 mM, and at higher Li^+ concentration when Mg^{++} levels are greater.

Because a great deal more is known about hormone secretion in the mouse condition for Li^+-induced inhibition of secretion were established. Chronic Li^+ feeding and acute Li^+ injection inhibited both DBC-AMP-stimulated release of ^{131}I and TSH stimulated release of ^{131}I. By use of preincubation with Li^+, inhibition of TSH stimulated ^{131}I release was demonstrated in a completely in vitro system. ^{131}I release and colloid droplets were decreased in parallel. Li^+ inhibition of thyroid secretion thus occurs also at a step between the generation of intracellular cyclic AMP and colloid droplet formation.

Honors and Awards: None

1. Berens, S. C., Bernstein, R. S., Robbins, J. and Wolff, J. Antithyroid effects of lithium. J. Clin. Invest. 49: 1357-1367, 1970.

2. Berens, S. C., Wolff, J. and Murphy, D. L. Lithium concentration by the thyroid. Endocrinology 87: 1085-1087, 1970.

3. Williams, J. A., Berens, S. C. and Wolff, J. Thyroid secretion in vitro: Inhibition of TSH and dibutyryl cyclic-AMP stimulated ^{131}I release by Li^+. Endocrinology 88: 1390-1396, 1971.

Serial No. NIAMD/CEB 23c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Thyroid Hormone Secretion

Previous Serial Number: none

Principal Investigators: J. A. Williams, M.D., Ph.D., J. Wolff, M.D.,Ph.D.

Other Investigator: None

Cooperating Units: None

Man Years

Total: 1 1/4
Professional: 1
Other: 1/4

Project Description:

The in vitro secretion of ^{131}I from prelabeled mouse thyroids was measured under conditions of TSH and cyclic nucleotide stimulation as well as inhibition by a variety of agents. TSH, 3',5'-cyclic AMP and dibutryl cyclic AMP all caused a dose dependent release of nonprotein ^{131}I superimposed on a constant basal amount of 19 S iodoprotein, confirming the usefulness of the Munro assay. Release of iodoprotein has been reduced to 2-6% of thyroidal ^{131}I over a 6 hr period of incubation with tissues showing good histologic preservation. Fluoride at concentrations above 3 mM caused release of iodoprotein and blocked the response to both TSH and cyclic AMP. One mM theophylline potentiated TSH and cyclic AMP induced release but higher concentrations released iodoprotein and 10 mM theophylline blocked the physiological response to TSH. These multiple actions help explain discrepancies in previous experimental findings with fluoride and theophylline and suggest caution in their use. Chlorpromazine inhibited nonprotein ^{131}I release by both TSH and dibutryl cyclic AMP but at higher concentrations released iodoprotein. Similar biphasic response curves were produced by the detergents sodium dodecyl sulfate and thymol. In contrast, cationic detergents released iodoproteins at lower concentrations than those at which inhibition of nonprotein ^{131}I release occurred, and polylysine released iodoprotein without interfering with TSH stimulation of nonprotein ^{131}I. It is concluded that thyroid membranes exposed to membrane stabilizers undergo typical stabilization-destabilization patterns characteristic of these agents. While all membranes are affected, the evidence suggests that inhibition of thyroid secretion occurs as a result of stabilization of the

apical cell membrane with prevention of colloid droplet formation.

In addition the role of external cations, the membrane potential, the Na^+ pump and ouabain in secretion are currently being studied.

We have also investigated the role of microtubules in the secretory process. Colchicine and other microtubule-active agents have been found to block the release, stimulated by either thyroid-stimulating hormone or by dibutyryl cyclic adenosine 3':5'-monophosphate, of ^{131}I from previously ^{131}I labeled mouse thyroid glands in vitro. The time and concentration characteristics of these inhibitors are consistent with their actions on microtubules in other systems. [^3H] colchicine was also shown to be bound to a soluble 6 S protein of bovine thyroid slices similar to the protein identified in other systems as a microtubular subunit. The demonstrated inhibition of colloid droplet formation and absence of an effect on thyroidal adenyl cyclase or cyclic 3':5'-phosphodiesterase suggests a colchicine-sensitive role for microtubules in colloid endocytosis in the thyroid gland.

Honors and Awards: None

Publications:

1. Williams, J. A. and Wolff, J.: Thyroid secretion in vitro: Multiple actions of agents affecting secretion. Endocrinology 88: 206-217, 1971.

2. Williams, J. A. and Wolff, J.: Possible role of microtubules in thyroid secretion. Proc. Nat. Acad. Sci. 67: 1901-1908, 1970.

PEDIATRIC METABOLISM BRANCH

In the past year the PMB has concentrated its investigations on genetic pancreatic diseases. The most common of these conditions is cystic fibrosis of the pancreas (CF), but familial hereditary pancreatitis (HP) has also been the subject of intensive study.

A. CYSTIC FIBROSIS OF THE PANCREAS

1. Study of the Basic Defect

The fibroblast in CF: In 1968 Danes and Bearn described cytoplasmic metachromasia in tissue culture fibroblasts obtained from skin biopsies of CF patients and of their parents. Much hope was raised that a quick solution to the problem of the basic defect in CF might be forthcoming by tissue culture studies.

A comprehensive investigation was undertaken, therefore, by the PMB in cooperation with several other laboratories to assess the morphologic and biochemical parameters of tissue culture fibroblasts from patients with CF. Tissue cultures were established by standard methods from skin punch biopsies obtained from known patients with CF, parents (obligate heterozygotes), and normal controls.

Morphologic studies to date have been unrewarding. It was not possible to reproduce different types of metachromasia postulated by Danes in CF fibroblasts. Ultrastructural studies up to now have shown no difference between CF fibroblasts and those from normal controls.

Matalon and Dorfman in 1968 reported a variable, but at times marked increase in total acid mucopolysaccharides (AMPS) in the fibroblasts of patients with CF with a normal distribution of fractions. However, Wiesmann and Neufeld, carrying out studies of the turnover of sulfated mucopolysaccharides in fibroblasts from 13 patients with CF found that the incorporation of this material differs only slightly, if at all, from the normal: these results are quite different from those in Hurler and Hunter cells. Although not directly related to tissue culture, studies on urinary excretion of acid mucopolysaccharides in 17 CF patients, 9 to 25 years of age, revealed these compounds in the urine to be essentially normal in the patients studies. This is in contrast to the distinctive patterns in urinary AMPS in most genetic mucopolysaccharidoses. The results of both of these investigations were thought to make a primary defect in mucopolysaccharide metabolism in CF very improbable.

However, because of the increase in the AMPS in the fibroblasts reported by Matalon and the excessive amount of viscous secretions that patients with this disorder exhibit, a study was undertaken to determine the function played by microtubules in normal and pathologic fibroblasts in the secretion of AMPS and whether there are any qualitative and/or quantitative differences between CF and normal fibroblasts in their response to colchicine which is known to disrupt these substructures and inhibit secretion. While these studies are in the preliminary stage, it does appear that the secretion of AMPS in the normal

fibroblasts is significantly influenced by the addition of colchicine to the medium whereas no effect is observed in the fibroblasts of patients with CF. It is too early to interpret these initial results.

Pallavicini et al. demonstrated that the glycogen content of fibroblasts from CF patients is significantly higher than that from normal control cells, although the increase seems to appear only after several days in culture. The glycogen was identified by various chemical and enzymatic methods. Cell density or the number of cells dividing had no measurable influence on the accumulation of the polysaccharide and in repeated determinations with cells from the same patients or controls glycogen levels behaved in a consistent manner. The reason for this accumulation has been explored by various methods. It was found that no block exists in the CF cells in glycolysis and that there is no difference in response between CF cells and those from normal controls to 2,4-dinitrophenol, phloridzin, epinephrin, insulin or osmolarity. Using C^{14} labeled glucose, there was no increase in the glucose in the CF cell lines as compared to controls. Variation in extracellular Na levels and adding ouabain to the medium in addition to the expected changes in the electrolyte composition inside the cells had a marked effect on the accumulation of glycogen which was always much more marked in the CF fibroblasts. While these experiments are still in course, there is as yet no good explanation as to the reasons for the glycogen accumulation in CF fibroblasts.

In conclusion, while the findings in tissue culture fibroblasts are of importance in showing that perhaps all cells in the body, and not only in the exocrine glands, are involved in the CF process and that there is abnormality in metabolism in CF fibroblasts, this defect has not been defined as yet. (Drs. Pallavicini, Neufeld, Taussig, di Sant'Agnese, NIAMD; Dr. Uhlendorf, DBS; Dr. Zweig, NHLI; Dr. Spicer, Univ. of S. Car., Charleston; Dr. Blanc, Columbia University, New York)

The "Cystic Fibrosis Factor": It was shown by Spock (1968) that a so-called "ciliary factor" exists in the serum of homozygotic patients and in the euglobulin fraction of heterozygotes that induces ciliary dyskinesia in the ciliated epithelium of rabbit tracheal explants. Subsequently many people, including ourselves, have tried to develop simpler assay systems by utilizing a variety of low animal forms (e.g., clam, oysters, etc.). However, this assay is fraught with pitfalls because of subjective interpretation as well as variation in the metabolism of the organisms used due to climatic conditions, seasonal variations, water temperature, etc..

About the same time Mangos (1968) demonstrated the presence in mixed saliva and sweat of CF patients (homozygotes) of a factor which inhibits Na reabsorption in an animal model (rat parotid). Subsequently, Kaiser in Switzerland (1970) was able to reproduce the same effects with sweat from CF patients in human sweat glands. We are trying to develop a simple assay system in inverted rat gut sacs incubated in the presence of CF and control saliva and studying the absorption of radioactive Na. In addition, alanine absorption is also studied in the same manner.

Although a significant number of salivas from patients with CF and controls were studied in this manner, it was not found that there is a significant difference in either sodium transport or alanine transport across the gut between the CF group and normal controls. These studies are being continued in an attempt to develop an easily reproducible and reliable assay for the so-called CF factor. If this were feasible it would open the way to chemical characterization of this factor, to ante-natal diagnosis, and to the determination of heterozygotes and therefore genetic counseling. (Drs. Taussig, Gardner, NIAMD)

Along somewhat similar lines, the sodium transport in the erythrocyte was studied in CF. Indeed it had been reported (Balfe et al., 1968) that of the major components of sodium outflux, the portion sensitive to ouabain and the one insensitive to ouabain but sensitive to ethacrynic acid were abnormal both in the homozygotes and the obligatory heterozygotes for the CF gene. This was studied quite extensively and no difference whatsoever was found in the various components between heterozygotes and normal controls, although it was found that a difference normally exists between normal males and normal females. This presumably accounts for the previously reported apparently erroneous findings, as this difference had not been taken into sufficient account. The ouabain-sensitive component of the sodium outflux was normal in all CF groups (homozygotes), but the ouabain-insensitive ethacrynic acid-sensitive appears to be abnormal in some of the groups under consideration.

The normal data from heterozygotes indicate that the erythrocyte sodium outflux cannot be used as a "genetic marker" for CF. The seemingly altered cation transport in non-exocrine tissue from males and normal females with CF is of uncertain significance and should be studied further in other tissues from such patients. (Drs. Lapey, Gardner, NIAMD)

Submaxillary saliva investigations: Gugler et al. (1967), from this laboratory, described in human submaxillary saliva a protein which precipitates in the presence of calcium (CaPP). The CaPP from normal human subjects was purified, its carbohydrate and amino acid composition determined, its minimal calculated molecular weight was 11,400, and several other parameters determined. This glycoprotein also contains 0.6% phosphorus and hydroxylysine in approximately the same molar quantity as glucose. CaPP from 6 normal subjects was compared to the same fraction from 6 patients with CF and a number of parameters studied. No consistent differences were found.

This represents the first time phosphoproteins have been found in submaxillary saliva and their role in the maintenance of integrity of tooth enamel deserves further study. In addition, in this study for the first time a purified and separated protein fraction in normal subjects was compared to that in patients with CF and properties were found to be similar. Because of the elevated Ca levels which obtain in the saliva of these patients, calcium forms bridges by electrostatic interaction with phosphate prosthetic groups of CaPP resulting in the precipitation and turbidity of submaxillary saliva in CF patients. (Drs. Boat, Pallavicini, NIAMD)

2. Metabolic Studies

A number of metabolic studies on patients with CF are either being completed or are now in progress. (Drs. Simopoulos, Kattwinkel, Taussig, di Sant'Agnese, Laster, NIAMD; Drs. Pak, Bartter, Henkin, NHLI)

Aldosterone metabolism: Aldosterone-renin system metabolism has never been accurately determined in CF despite the fact that these patients show inability to retain salt in their sweat, leading to serious and at times fatal complications through cardiovascular collapse. Five CF patients, 13 to 21 yrs of age, were therefore studied on an air conditioned metabolic unit on constant dietary regimens with varying sodium content. It was found that the aldosterone secretion rate and renin values were slightly elevated, but responded normally to decreased oral sodium intake and postural changes. ASR did not suppress as expected on the high sodium intake. It was felt that these patients had a state of hyperaldosteronism secondary to increased renin release, probably as an adaptation to frequent excessive sodium losses by the sweat and consequent extracellular changes.

Calcium metabolism and parathyroid function in CF: Demineralization of bones to a varying degree has been reported in CF as also have some calcium level abnormalities of some biologic fluids (submaxillary and parotid saliva), although serum Ca and P levels are normal. Therefore, 5 CF patients, age 14 to 23 yrs, were studied by calcium metabolic balances as well as calcium infusion tests. Bone density in 21 patients was evaluated by a roentgenographic computerized method and the results are still being processed. Only one of the 5 patients studied on metabolic balances was in slightly negative Ca balance while the 4 others were in slightly positive balance at the time of study. Results of the calcium infusion studies showed a consistent increase in the urinary cyclic AMP and a lack of the normal fall in the urinary phosphorus. These findings are consistent with either non-suppressible hyperparathyroid function or with the failure of end organ response. It was thought at first that this state of hyperparathyroidism might be secondary to the malabsorption all of these patients have, but results of Ca^{47} absorption tests on 2 patients out of 5 so far indicate that this may not be the case. We do not have an explanation as yet for these findings and further studies are planned.

Copper and zinc metabolism in CF: It has been suggested by several investigators that copper metabolism in CF is abnormal. Therefore, the concentrations of copper and zinc were studied in serum and urine of CF patients and controls and attempts made to evaluate these metals in saliva and sweat. Preliminary results indicate that the serum and urniary copper in CF is normal, but serum zinc is significantly decreased while the urinary zinc is significantly increased. These results are being evaluated and further studies are being performed.

Malabsorption in older patients: Pancreatic function and fecal fat and nitrogen losses are being assessed on a large number of adolescents and young adults with CF. It was found that steatorrhea and azotorrhea are still very marked in most older patients with CF, although intestinal symptomatology is markedly improved compared to childhood. The extent of fecal loss does not

correlate with the state of nutrition, age, growth, severity of pulmonary disease or age at diagnosis.

3. Studies of the Complications and Treatment of CF

Treatment of malabsorption: Studies are underway of the relative effect and efficacy of pancreatic replacement therapy (cotazym), oral administration of arginine which has been advocated by some groups, and the addition of sodium bicarbonate to the pancreatic extract preparations because of the absence of pancreatic juice in patients with CF and consequent difference in the duodenal pH. While the studies are still underway, preliminary evaluation would indicate that arginine is not effective in reducing the fat and nitrogen fecal losses while cotazym does cause some degree of improvement and cotazym with the addition of sodium bicarbonate causes additional marked improvement at least in some of the patients under study.

Treatment of the chronic pulmonary disease: New ways of approaching some of the pulmonary complications of CF, notably pneumothorax and massive hemoptysis, are being explored by surgical-medical means. The natural history of the lung disease in CF with special relation to these complications and their possible prevention is being studied further. New antibiotic therapy of the pulmonary component of this disease is being further evaluated with specific attention being paid to the anti-Pseudomonas agents, gentamicin and carbenicillin, and the potent anti-Staphylococcus agent, clindamycin.

B. OTHER GENETIC PANCREATIC DISORDERS

This Branch has had an interest and is investigating other genetic pancreatic disorders, such as familial pancreatitis, pancreatic insufficiency and bone marrow dysfunction, and others. Studies on familial pancreatitis have been carried to some degree of completion.

Hereditary pancreatitis: Hereditary pancreatitis (HP) has been reported in the last few years in 13 relatively small kindred primarily in this country and is characterized by chronic relapsing pancreatitis leading to pancreatic insufficiency, pancreatic calcification, and at times diabetes and abdominal carcinoma. In 3 of the original families lysine-cystine aminoaciduria was present in some members, regardless of pancreatic involvement.

We have studied two large unrelated kindred with HP originally from isolated mountain communities. The West Va. kindred with 105 members and the Tennessee kindred with 115 members with a combined total of 34 definite or suspected cases of pancreatitis. In both kindred fecal fat, pancreatic enzymes, and serum amylase and lipase were assessed. Serum lipids and parathyroid function by calcium infusion were normal. All urinary amino acids were determined in selected patients and relatives and were normal in all instances. HP usually started in early infancy although the diagnosis was generally not made until the patients presented as adolescents or young adults with pancreatic calcifications. Abdominal carcinoma occurred in several patients, emphasizing its relation to pancreatic calcifications. The mode of genetic transmission suggests an autosomal dominant with incomplete and

variable penetrance. The etiology of HP is obscure but the factors usually indicted in the case of other types of chronic relapsing pancreatitis (e.g., alcohol, gallstones) do not seem to be operative. A structural defect may conceivably have been at fault in two of the families described in the literature, but presumably not in the others.

It was concluded that HP is probably the most common cause for relapsing pancreatitis in childhood and should always be considered in the differential diagnosis of recurrent abdominal pain and abdominal calcification in childhood. Its frequency in adults is unknown but may be more common than previously thought. It is speculated that at least three different genetic types of HP exist: with and without aminoaciduria and one kindred described in France which has a clinical course, complications, and pathologic findings which differed from those of the other reported families. (Drs. Kattwinkel, di Sant'Agnese, Pallavicini, Laster, NIAMD)

Serial No. NIAMD-PMB-1c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemical and Immunologic Studies of Submaxillary Saliva in Cystic Fibrosis.

Previous Serial No.: Same

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: T. F. Boat, M.D., J. Charles Pallavicini, Ph.D., Phyllis Jen, B.S., NIAMD.

Cooperating Units: Frederick Bettelheim, M.D., Dept. of Biochemistry, Adelphi University, Garden City, N.Y.

Man Years: Total: 0.75
Professional: 0.50
Other: 0.25

Project Description:

Objectives:

Two separate studies were undertaken:
1. Immunologic investigation of salivary proteins with the object of detecting differences both in the "specific" salivary proteins and in the salivary albumin and IgA between patients with cystic fibrosis and normal individuals in the behavior of the protein as related to flow rate.

2. It was the object of a separate project to define the physico-chemical properties of the calcium precipitable proteins, determining the relationship of CaPP to other submaxillary salivary proteins, to define the specific conditions which contribute to a change of CaPP *in vitro* and to compare the submaxillary saliva of CF patients and controls.

Methods Employed and Patient Material:

Submaxillary saliva was collected from male and female cystic fibrosis patients and normal controls between 10 and 30 years of age. The secretions were collected by a modified Schneyer technique which segregates the methylmethacrylate constructed individually. A variety of chemical methods and of immunodiffusion techniques were used in these investigations.

Major Findings:

Both of these projects are now completed and in the process of being written up in several different papers to be submitted for publication.

Results can be briefly summarized. In the first investigation phosphoproteins were shown for the first time to be present in submaxillary saliva and have an important role in the maintenance of integrity of tooth enamel. In addition, in this study for the first time a purified and separated protein fraction in normal subjects as compared to that in patients with CF and controls was found to be similar. An explanation was given of the mechanism of the precipitation process which obtains in submaxillary saliva in CF and may be similar to the precipitation of secretions throughout the body giving rise to obstruction which results in virtually all of the clinical manifestations of this disease. In addition, the finding of glucose-containing oligosaccharides, presumably linked to hydroxylysine in one of these proteins (CaPP) indicates that this particular chemical unit appears more frequently than had been previously suspected. Up to the present time glucose-containing oligosaccharides had only been demonstrated in collagen and basement membrane protein (Spiro, 1969).

A quantitative comparison between normal and CF saliva "specific" proteins is difficult because of significant quantitative as well as qualitative individual variations. The IgA levels in CF saliva were significantly increased as compared to normal and the behavior of both albumin and IgA increasing flow rate was found to be distinctive.

Honors and Awards: None

Publications: Several manuscripts in preparation.

Serial No. NIAMD-PMB-2c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Tissue Culture Investigations and Studies of Urinary AMPS Excretion.

Previous Serial No.: Same

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: J. Charles Pallavicini, Ph.D., Lynn M. Taussig, M.D., E. Neufeld, Ph.D., NIAMD; William Uhlendorf, Ph.D., DBS; G. Constantopoulos, Ph.D., Anatole Dekaban, M.D., NINDS.

Cooperating Units: S. S. Spicer, M.D., University of S. Car., Charleston, S.C.

Man Years: Total: 1.50
Professional: 1.00
Other: 0.50

Project Description:

Objectives:

In 1968 Danes and Bearn described cytoplasmic metachromasia in tissue culture fibroblasts obtained from skin biopsies of cystic fibrosis patients and of their parents. Much hope was raised that a quick solution to the problem of the basic defect in CF might be forthcoming by tissue culture studies. A comprehensive investigation was undertaken, therefore, by the Pediatric Metabolism Branch in cooperation with several other laboratories to assess the morphologic and biochemical parameters of tissue culture fibroblasts from patients with CF.

Methods Employed and Patient Material:

Patient material consisted of known patients with cystic fibrosis, parents (obligate heterozygotes), and normal controls. Skin punch biopsies were obtained and cultured in Eagle's medium reinforced with 10% fetal calf serum. Fibroblasts were subcultured and either grown in quantity and harvested for biochemical studies or grown on glass coverslips for fixation and staining, or grown in flasks for subsequent harvesting and light microscopy.

For colchicine studies the fibroblasts were exposed to various concentrations of this drug for various periods of time. AMPS synthesis and secretion

were measured by incorporation of S_{35} in AMPS. The cells were harvested, protein determinations made, cells washed and then counted, and media dialyzed and then counted. Effects of colchicine on the tissues were evaluated by light microscopy. The effects, if any, of 17 beta-estradiol on AMPS secretion in tissue culture are now also being evaluated.

For evaluation of AMPS excretion in cystic fibrosis, these compounds were isolated by both CPC precipitation and Ecteola columns in repeated 24-hour urine collections from 17 CF patients 9 to 25 years of age. The uronic acid content was determined by the carbazole and orcinol methods, paper electrophoresis, n-sulfation and elution diagrams on Sephadex G-200 columns were performed on the isolated AMPS.

Major Findings:

Morphologic studies to date have been unrewarding. It was not possible to reproduce the different types of metachromasia observed by Danes in CF fibroblasts. Ultrastructural studies up to now have shown no difference between CF fibroblasts and those from normal controls.

Matalon and Dorfman in 1968 reported a variable, but at times marked increase in total acid mucopolysaccharides (AMPS) in the fibroblasts of patients with CF with a normal distribution of fractions. However, Wiesmann and Neufeld, carrying out studies of the turnover of sulfated mucopolysaccharides in fibroblasts from 13 patients with CF found that the incorporation of this material differs only slightly, if at all, from the normal: these results are quite different from those in Hurler and Hunter cells. Studies on urinary excretion of acid mucopolysaccharides in 17 CF patients, 9 to 25 years of age, revealed these compounds in the urine to be essentially normal in the patients studied. This is in contrast to the distinctive patterns in urinary AMPS in most genetic mucopolysaccharidoses. The results of both of these investigations were thought to make a primary defect in mucopolysaccharide metabolism in CF very improbable.

However, because of the increase in the AMPS in the fibroblasts reported by Matalon and the excessive amount of viscous secretions that patients with this disorder exhibit, a study was undertaken to determine the function played by microtubules in normal and pathologic fibroblasts in the secretion of AMPS and whether there are any qualitative and/or quantitative differences between CF and normal fibroblasts in their response to colchicine which is known to disrupt these substructures and inhibit secretion. While these studies are in the preliminary stage, it does appear that the secretion of AMPS in the normal fibroblasts is significantly influenced by the addition of colchicine to the medium whereas no effect is observed in the fibroblasts of patients with CF. It is too early to interpret these initial results.

Pallavicini et al. demonstrated that the glycogen content of fibroblasts from CF patients is significantly higher than that from normal control cells, although the increase seems to appear only after several days in culture. The glycogen was identified by various chemical and enzymatic methods. Cell density or the number of cells dividing had no measurable influence on the

accumulation of the polysaccharide and in repeated determinations with cells from the same patients or controls glycogen levels behaved in a consistent manner. The reason for this accumulation has been explored by various methods. It was found that no block exists in the CF cells in glycolysis and that there is no difference in response between CF cells and those from normal controls to 2,4-dinitrophenol, phloridzin, epinephrine, insulin or osmolarity. Using C^{14} labeled glucose there was no increase in the glucose in the CF cell lines as compared to controls. Variation in extracellular sodium levels and adding ouabain to the medium in addition to the expected changes in the electrolyte composition inside the cells had a marked effect on the accumulation of glycogen which was always much more marked in the CF fibroblasts. While these experiments are still in course, there is as yet no good explanation as to the reasons for the glycogen accumulation in CF fibroblasts.

Significance to Biomedical Research:

The findings in tissue culture in cystic fibrosis are important in two respects. They represent the first available experimental model outside the patients' bodies, and it now appears possible that in reality all cells are involved in the process leading to the manifestations of this generalized disease, while in the past CF was felt to be limited to the exocrine glands.

Honors and Awards: None

Publications:

Pallavicini, J.C., Wiesmann, U., Uhlendorf, W.B., and di Sant'Agnese, P.A.: Glycogen content of tissue culture fibroblasts from patients with cystic fibrosis and other heritable disorders. J. Pediat. 77:280-284, 1970.

Wiesmann, U., and Neufeld, E.F.: Metabolism of sulfated mucopolysaccharide in cultured fibroblasts from cystic fibrosis patients. J. Pediat. 77:685-690, 1970.

Constantopoulos, G., Dekaban, A.S., Lapey, A., and di Sant'Agnese, P.A.: Urinary acid mucopolysaccharides in cystic fibrosis. J. Pediat. 78:806-811, 1971.

Serial No. NIAMD-PMB-3c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in Familial Inherited Pancreatitis.

Previous Serial No.: None

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: John Kattwinkel, M.D., J. Charles Pallavicini, Ph.D., Leonard Laster, M.D., NIAMD.

Cooperating Units: Digestive and Hereditary Diseases Branch, NIAMD.

Man Years: Total: 0.75
Professional: 0.75
Other: 0

Project Description:

Objectives:

Two large kindred are being studied with familial inherited pancreatitis, a relatively new genetic pancreatic disease characterized by chronic relapsing pancreatitis leading to pancreatic insufficiency, pancreatic calcification, and at times diabetes and abdominal carcinoma.

Methods Employed and Patient Material:

We have studied two large unrelated kindred with hereditary pancreatitis (HP) originally from isolated mountain communities. The West Va. kindred with 105 members and the Tennessee kindred with 115 members with a combined total of 35 definite or suspected cases of pancreatitis. In both kindred fecal fat, pancreatic enzymes, and serum amylase and lipase were assessed. Serum lipids and parathyroid function (by calcium infusion) were normal. All urinary amino acids were determined in selected patients and relatives, and were normal in all instances. HP usually started in early infancy although the diagnosis was generally not made until the patients presented as adolescents or young adults with pancreatic calcifications. Abdominal carcinoma occurred in several patients, emphasizing its relation to pancreatic calcifications. The mode of genetic transmission suggests an autosomal dominant with incomplete and variable penetrance. The etiology of HP is obscure but the factors usually indicted in the case of other types of chronic relapsing pancreatitis (e.g., alcohol, gallstones) do not seem to be operative. A structural defect may conceivably have been at fault in two of the families described in the literature, but presumably not in the others.

It was concluded that HP is probably the most common cause for relapsing pancreatitis in childhood and should always be considered in the differential diagnosis of recurrent abdominal pain and abdominal calcification in childhood. Its frequency in adults is unknown but may be more common than previously thought. In three of the original families lysine-cystine aminoaciduria was present in some members regardless of pancreatic involvement; however, this has not been found in any of the families described since then.

It is speculated that at least three different genetic types of HP exist: with and without aminoaciduria and one kindred described in France which has a clinical course, complications, and pathologic findings which differed from those of the other reported families.

Significance to Biomedical Research:

The further definition of the genetic transmission, clinical and laboratory manifestations, and of the prognostic implications of this relatively new disease will have important implications in pediatric and adult gastroenterology. It will further our knowledge of the relatively unknown and new group of pancreatic disorders of genetic origin.

Honors and Awards: None

Publications: None

Serial No. NIAMD-PMB-4c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemical Studies in Cystic Fibrosis.

Previous Serial No. None

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: Lynn M. Taussig, M.D., Jerry D. Gardner, M.D., NIAMD: Robert O. Wolf, D.D.S., NIDR.

Cooperating Units: Digestive and Hereditary Diseases Branch, NIAMD; Oral Medicine and Surgery Branch, NIDR.

Man Years: Total: 1.00
Professional: 0.75
Other: 0.25

Project Description:

Objectives:

1. The basic defect in cystic fibrosis remains unknown. Recently several investigators have described the presence of two factors in saliva, sweat, and serum of patients: a ciliary inhibitory factor and a factor which inhibits sodium reabsorption across various membranes. Since there is no easy, reproducible assay for either factor, studies were begun to develop a biologic assay for the sodium inhibitory factor.

2. Balfe et al., 1968 reported that some of the major components of sodium outflux in erythrocytes were abnormal both in the homozygotes and the obligatory heterozygotes for the CF gene. It was planned to repeat these experiments and to either confirm or deny this potentially important observation.

3. As of now there is no method available to make a prenatal diagnosis of CF which would be of obvious practical importance in the control of the disease. Over the past few years amniotic fluid has become a valuable source of material for the prenatal diagnosis of various congenital diseases, but nothing is known about the fluid surrounding fetuses who have a high probability of having CF. As 90% of patients with CF have pancreatic insufficiency at birth as well as an abnormality in the sodium and chloride content of sweat, a study was undertaken to evaluate these components in amniotic fluid.

Methods Employed:

Biologic assay for the sodium inhibitory factor in CF: Rat intestine was used and the uptake of radioactive sodium measured. Double isotope studies were performed with labeled carbon used as a volume marker. Two different assays were established with the tissue incubated in either normal or cystic mixed saliva and the results of sodium uptake compared. Since about 15% of sodium uptake is coupled to alanine absorption and since all of this amino acid absorbed is coupled to sodium uptake, an assay system was set up to measure the uptake of carbon labeled alanine across resin in the presence of either normal or cystic saliva.

Erythrocyte sodium fluxes: Sodium content was measured flame photometrically. Sodium fluxes were measured using ^{24}Na. The effect of alterations in the cation composition of the incubation medium and of aldosterone (2×10^{-6}M), ouabain (10^{-4}M) or ethacrynic acid (10^{-3}M) on sodium influx and sodium outflux were measured.

Amniotic fluid studies: Amniotic fluid was obtained from mothers who had previously given birth to children with cystic fibrosis ("subjects") and compared with that obtained at delivery, amniocentesis, or abortion from mothers with no family history of CF ("controls"). Amylase and its numerous isoenzymes were studied in detail using acrylamide gel, starch gel, and agar gel electrophoresis as well as direct starch-iodine determination of amylase levels. Other pancreatic enzymes including trypsin, chymotrypsin, and carboxypeptidase A and B were studied.

Major Findings:

Biologic assay for the sodium inhibitory factor in cystic fibrosis: Rat gut sacs when incubated in the presence of cystic saliva appeared in all cases but one to absorb less radioactive sodium than did sacs incubated in the presence of normal saliva. The results, however, were not statistically significant. Preliminary studies reveal that when rat intestine is incubated in the presence of cystic saliva there is an enhancement of alanine uptake compared to intestine incubated in the presence of normal saliva.

Erythrocyte sodium transport was studied in 26 normals, 25 patients with CF, and 20 parents of patients with CF. No difference was found in the various components between heterozygotes and normal controls, although it was found that a difference normally exists between normal males and normal females. This presumably accounts for the previously reported apparently erroneous findings, as this difference had not been taken into sufficient account. The ouabain-sensitive component of the sodium outflux was normal in all CF groups (homozygotes), but the ouabain-insensitive ethacrynic acid-sensitive appears to be abnormal in some of the groups under consideration.

The normal data from heterozygotes indicate that the erythrocyte sodium outflux cannot be used as a "genetic marker" for CF. The seemingly altered

cation transport in non-exocrine tissue from males and normal females with CF is of uncertain significance and should be studied further in other tissues from such patients.

Amniotic fluid studies: These studies are still preliminary. Little is known about the source and nature of amniotic fluid amylase. Amniotic fluid from "control" mothers was first studied and it was found that amylase was present in concentrations slightly below normal serum levels and is composed of three distinct isoenzymes. The isoenzyme pattern is being compared to those of salivary, duodenal fluid, and placental homogenate amylases. So far it appears that the amniotic fluid pattern of this enzyme seems to be distinct from the other three sources. Amniotic fluid from "subject" mothers is being obtained and the levels and isoenzyme patterns of the amylase determined.

Significance to Biomedical Research:

Development of a simple assay for the circulating"CF Factor" is of great importance for the detection of heterozygotes, genetic counseling and antenatal diagnosis of CF, in addition to furnishing a tool for further laboratory studies of the basic defect of this disorder.

Honors and Awards: None

Publications:

Lapey, A. and Gardner, J. D.: Abnormal erythrocyte sodium transport in cystic fibrosis. Pediat. Res., in press.

Serial No. NIAMD-PMB-5c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Metabolic Studies in Cystic Fibrosis.

Previous Serial No.: Same

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: John Kattwinkel, M.D., Artemis P. Simopoulos, M.D. (guest worker), Leonard Laster, M.D., NIAMD.

Cooperating Units: Endocrinology Branch, NHLI.

Man Years: Total: 2.50
Professional: 1.50
Other: 1.00

Project Description:

Objectives:

Comprehensive metabolic studies were undertaken with the object of clarifying the physiopathology of cystic fibrosis and thus permitting a more effective and rational approach to prognosis and treatment.

Methods Employed and Patient Material:

Aldosterone metabolism: Five CF patients, 13 to 21 years of age, were studied on an air conditioned metabolic unit on constant dietary regimens with 9, 109, and 240 mEq/24 hours sodium intake for 8-day periods. During each 8-day period of different sodium balance, aldosterone secretion rate (ASR) and plasma renin activity were measured; the latter in both supine and upright positions.

Calcium metabolism: Five CF patients, age 14 to 23 years, were placed for 17 to 22 days on metabolic balance. These 5 CF patients were then placed for 4 days on a liquid diet with 400 mg of calcium and 900 mg of phosphorus per day. On the third day calcium infusion (15 mg Ca/kg body weight) was performed and various determinations in the blood and urine including phosphorus clearance and urinary cyclic AMP were performed. Blood was also collected for parathormone levels. Calcium intestinal absorption was assessed by means of Ca^{47} absorption tests.

Copper and zinc metabolism: Copper and zinc were determined in serum and in 24-hour urine collections in 16 patients with CF, 12 to 27 years of age, by atomic absorption spectrophotometry.

Malabsorption studies: With the subjects on metabolic diets, fecal fat and nitrogen were determined on 3 consecutive 3-day stool pools with measured dietary intake in 20 patients with CF, ranging from 12 to 29 years of age. All pancreatic enzymes, proteolytic ones as well as lipase and amylase were determined by duodenal assay and a number of other chemical parameters were obtained simultaneously.

Major Findings:

Aldosterone metabolism: Aldosterone-renin system metabolism has never been accurately determined in CF despite the fact that these patients show inability to retain salt in their sweat, leading to serious and at times fatal complications through cardiovascular collapse. Five CF patients, 13 to 21 years of age, were therefore studied on an air conditioned metabolic unit on constant dietary regimens with varying sodium content. It was found that the aldosterone secretion rate and renin values were slightly elevated, but responded normally to decreased oral sodium intake and postural changes. ASR did not suppress as expected on the high sodium intake, It was felt that these patients had a state of hyperaldosteronism secondary to increased renin release, probably as an adaptation to frequent excessive sodium losses by the sweat and consequent extracellular volume changes.

Calcium metabolism and parathyroid function in CF: Demineralization of bones to a varying degree has been reported in CF as also have some calcium level abnormalities of some biologic fluids (submaxillary and parotid saliva), although serum Ca and P levels are normal. Therefore, 5 CF patients, age 14 to 23 years, were studied by calcium metabolic balances as well as calcium infusion tests. Bone density in 21 patients was evaluated by a roentgenographic computerized method and the results are still being processed. Only one of the 5 patients studies on metabolic balances was in slightly negative Ca balance while the 4 others were in slightly positive balance at the time of study. Results of the calcium infusion studies showed a consistent increase in the urinary cyclic AMP and a lack of the normal fall in the urinary phosphorus. These findings are consistent with either non-suppressible hyperparathyroid function or with the failure of end organ response. It was thought at first that this state of hyperparathyroidism might be secondary to the malabsorption all of these patients have, but results of Ca^{47} absorption tests on 2 patients out of 5 so far indicate that this may not be the case. We do not have an explanation as yet for these findings and further studies are planned.

Copper and zinc metabolism in CF: It has been suggested by several investigators that copper metabolism in CF is abnormal. Therefore, the concentrations of copper and zinc were studied in serum and urine of CF patients and controls and attempts made to evaluate these metals in saliva and sweat. Preliminary results indicate that the serum and urinary copper in CF is normal. Serum zinc is significantly decreased while the urinary zinc is significantly

increased. These results are being evaluated and further studies are being performed.

Malabsorption in older patients: Pancreatic function and fecal fat and nitrogen losses are being assessed on a large number of adolescents and young adults with CF. It was found that steatorrhea and azotorrhea are still very marked in most older patients with CF, although intestinal symptomatology is markedly improved compared to childhood. The extent of fecal loss does not correlate with the state of nutrition, age, growth, severity of pulmonary disease or age at diagnosis.

Also being studied as part of this project are the relative effect and efficacy of pancreatic replacement therapy (cotazym); oral administration of arginine, which has been advocated by some groups, and the addition of sodium bicarbonate to the pancreatic extract preparations because of the absence of pancreatic juice in patients with CF and consequent difference in the duodenal pH. Preliminary evaluation would indicate that arginine is not effective in reducing the fat and nitrogen fecal losses. Cotazym does cause some degree of improvement and cotazym with the addition of sodium bicarbonate causes additional marked improvement at least in some of the patients under study.

Significance to Biomedical Research:

As the basic defect in cystic fibrosis is still not known, it is obviously of importance to define further the endocrinology status of these patients. There are many tests which indicate the aldosterone metabolism as well as parathyroid function and calcium metabolism are not normal, but the significance of these findings cannot be interpreted as yet.

The metabolic studies relating to nutrition are of practical importance in improving the prognostic, diagnostic, and treatment methods in this disease. In addition, they are of interest to gastroenterology in indicating the relative importance of pancreatic secretion through the digestive processes in relation with age.

Honors and Awards: None

Publications:

Simopoulos, A. P., Lapey, A., Boat, T. F., di Sant'Agnese, P. A., and Bartter, F. C.: The renin-angiotensin-aldosterone system in patients with cystic fibrosis. Pediat. Res., in press.

di Sant'Agnese, P. A.: Cystic fibrosis and other genetic pancreatic diseases in childhood. In Beck, I. T. and Sinclair, D. (Eds.): The Exocrine Pancreas, London, J & A Churchill, 1971, pp. 227-246.

I. Study of Blood Coagulation and Diseases of Hemmorrhage and Thrombosis.

1. Attempts to identify old and new platelets.

There are numerous reports in the literature suggesting that platelets change their size, specific gravity and metabolism as they age. Because ability to identify old and new platelets would be helpful in differentiating between diseases of excess distruction and lack of production, we attempted to confirm these findings but were unable to do so on the basis of morphologic, physical, or biochemical characteristics. Specifically, by separating platelets into heavy and light populations we found that the two populations were of similar size and that their production of protein, lactate, CO_2 from C^{14}-labeled carbohydrate, and lipid from radioactive precursors were indistinguishable. Furthermore these two platelet populations separable by sedimentation reacted quantitively in the same way in aggregation phenomenon and in response to various agents that are known to agument or inhibit adenylcyclase and cyclic AMP levels. The two populations of platelets also survived the same period of time in vivo. These findings suggest that the age of platelets can not be discerned by current techniques. (Drs. N. Raphael Shulman, Richard Counts and Arthur Silvergleid).

2. Platelet aggregation.

In attempting to determine why aspirin inhibits aggregation we found that most parameters of platelet metabolism including protein synthesis, lactate production, and formation of CO_2 from carbohydrate were normal but that lipid synthesis was decreased. In addition, it appeared that aspirin decreased nucleotide release caused by aggregating agents. These effects were noted in platelets treated in vitro with aspirin as well as in platelets from individuals on high doses of aspirin. (Drs. Richard Counts, Carla Knepp and N. Raphael Shulman).

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In previous years we have studied cyclic AMP, adenylcyclase and phosphorylase activity with respect to platelet aggregation. A close relationship was found between inhibition of aggregation and those agents that increase intracellular cAMP and converse between enhancement of aggregation and decrease in cAMP. It is generally assumed that the final common pathway of platelet aggregation is ADP released prior to platelet aggregation. It has been considered that platelet granules contain ADP in sufficient amounts to initiate aggregation. We have found that a varity of substances such as poly-cations and poly-amino acids are capable of aggregating platelets without causing release of ADP. Moreover we found that ADP does not induce aggregation of platelets under the same circumstances that agents such as thrombin, collagen and epinephrine cause aggregation although these agents supposedly act through and ADP-dependent mechanism. Thus ADP does not appear to be the final common pathway of platelet aggregation and it appears that some other aggregating substance is released from platelets. These findings depended on our developing a system of aggregation involving washed platelets in a defined medium. (Drs. Richard Counts, Carla Knepp and N. Raphael Shulman).

4. Purification of factor VIII (antihemophilic factor).

Work has continued on further purification of factor VIII by proteolytic digestion followed by gel exclusion elution and electrolytic focusing. The protein factor has been found to contain carbohydrate and lipid but no phospholipid as suggested by some workers. The factor is of unusually large molecular weight (10^6) and consists of 4 to 5 subunits discernable on SDS acrylamide gels. It has been found that some hemophiliacs contain a similar molecule in their plasma, suggesting that some hemophiliacs contain a functionally inactive molecule that is otherwise similar to the normal factor VIII. (Drs. Sally Marchesi and N. Raphael Shulman).

5. Thrombaesthenia.

This rare hemaragic disorder involves an abnormality in platelets that has not yet been defined. Patients have infinite bleeding time but normal platelet counts and the platelets appear morphologically normal. We found that the overall protein, carbohydrate and lipid metabolism of these platelets was normal but their function in aggregation was highly abnormal. None of the known aggregating agents such as thrombin, epinephrine ADP or collagen induced aggregation. However the platelets responded noramlly to these aggregating agents as far release of intracellular material was concerned. Since platelet aggregation appears to be a consequence of the release reaction and a surface phenomenon involving substances that are released, it appears that thrombaesthenic patients may have an abnormaility in platelet membranes that prevents response to aggregation agents. Of special interest was the fact that thrombaesthenic platelets interfered with reactions of normal platelets. Mixtures of the two types gave abnormal results in aggregation and clot retraction retraction reactions. This interference phenomenon also occured in vivo, for platelet transfusions did not fully correct the bleeding time of thrombaesthenic patients unless normal platelets were supplied in two-fold greater concentration than the patients own platelets. Further studies are in progress on the electromicrographic appearence of thrombaesthenic platelets as well as on a comparison of the effects of various agents that bypass the release reaction in aggregating platelets. (Drs. Arthur Silvergleid, Richard Counts, Cecil Coleman and N. Raphael Shulman).

6. Prothrombin conversion.

Following last years lead on the possible existence of an intermediate between prothrombin and thrombin that could be defined physicochemically when prothrombin is converted by factor X in a purified system, we have attempted to isolate the intermediate by affinity columns, SDS acrylamide gel electrophoresis, and by DEAE cellulose chromotography. All of these techniques result in identification of a third substance in addition to prothrombin and thrombin, present during prothrombin conversion. It is this "intermediate" substance that appears to combine with soy bean trypsin inhibitor when clotting is inhibited by this proteolytic inhibitor. The prothrombin derivative appears to be involved in homeostatic control of clotting and should be helpful in defining new factors that accellerate or inhibit intravascular coagulation. (Drs. Richard Counts, Cecil Coleman and N. Raphael Shulman).

II. Study of the Immunology of Blood Cell Deficiencies.

1. A new test for viral hepatitis.

We developed a hemagglutination test for the antibody of long incubation hepatitis. This test is approximately one thousand times more sensitive than previous tests and as sensitive as the radioimmunoassay which was developed by others during the past year. Our hemagglutination test however is extremely simple, does not depend on any special reagent, and takes approximately 15 minutes rather than the several days radioimmunoassay takes to complete. With this test we have been able to detect antibodies in about 60% of patients following long incubation hepatitis. Antibodies frequently arise during the incubation period of the disease, and disappear at the time that viral antigen appears in the blood only to return later in the recovery period. Antibodies remain in the circulation for a matter of months or years thereafter but disappear in all but about 25% of cases after a year following acute hepatitis. With the new hemagglutination technique as many as 25% of normal blood donors have been found to have an anti-hepatitis antibody.

The same general technique of hemagglutination can be used to detect antigen by hemagglutination inhibition. This technique was found to be as sensitive as the most sensitive techniques available for detecting antigen. It compares favorably with complement fixation but is more rapid and simpler. (Drs. Girish Vyas and N. Raphael Shulman).

2. A fecal antigen in hepatitis.

In screening patients whose blood was positive for the hepatitis associated antigen (HAA) it was found that a high percent of the patients have in their stools a substance that would precipitate with an antibody present in the serum of approximately 10% of hepatitis patients. The antigen was detected in a filtered saline extract of stool and this was used to develop an antibody in rabbits. It appears that the stool antigen is present in most cases of HAA-positive hepatitis. The antigen however is much smaller than HAA and varies from greater than 300,000 M. W. to the size of albuim, possibly reflecting degredation in the GI tract. Whether the antigen is in any way related to the virus of long incubation hepatitits or whether it represents a helper or concurrent viral infection involving the gastrointestinal tract is not yet clear. (Drs. N. Raphael Shulman and Carla Knepp).

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There has been conflict in the literature concerning the frequency of finding HAA in patients with biliary cirrhosis. One group of workers have claimed that 90% of their patients with biliary cirrhosis have HAA whereas we have not been able to detect this antigen in this antigen in this disease. In a large series of over a 100 patients with biliary cirrhosis studied in association with Dr. Klatskin, at Yale, we screened for HAA and anti-HAA by three of the most sensitive test available and found the frequency of positive results no greater than in control groups. Biliary cirrhosis which by some is considered a complication of acute hepatitis therefore appears to be unrelated

to hepatitis as a result of our studies. (Drs. Gerald Klatskin and N. Raphael Shulman).

4. Effects of marcoglobulins on platelet function.

We have attempted to define the inhibitory action of macroglobulins on platelet aggregation. A series of macroglobulins obtained from patients with lymphoma or Waldenstrom hypergammaglobulinemia were found to inhibit platelet aggregation only if they had antigammaglobulin properties. Further studies showed that the macroglobulin antigamma reaction resulted in formation of complexes which were absorbed onto platelet surfaces. The macroglobulin did not attach to gammaglobulin that has been already absorbed on platelets nor did the macroglobulins absorb first on platelets, but only the complete complex was absorbed. Thus both types of gammaglobulin have to be present at the same time for inhibition of platelet function. This phenomenon has general implications in that it clearly documents destructive effects of the antigen-antibody complexes and suggest that some of the other manifestations such as nephritis and arthritis in certain hypergammaglobulinemias result from immune complexes. (Drs. Arthur J. Silvergleid and N. Raphael Shulman).

Serial No. NIAMD-CHB-1c
1. Clinical Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Study of Blood Coagulation and Diseases of Hemmorrhage and Thrombosis.

Previous Serial Number: SAME

Principal Investigator: Dr. N. Raphael Shulman

Other Investigators: Dr. Richard B. Counts
Dr. Arthur Silvergleid

Cooperating Units: Dr. Sally Marchesi, CC, CP

Man Years

Total:	3.5
Professional:	2.5
Other:	1.0

Project Description:

Objectives:

Study of the reactions and interactions of coagulation factors *in vitro* and *in vivo* to define further the nature of the blood coagulation mechanism, and to determine factors of significance in the pathogenesis of diseases of hemorrhage and thrombosis and develop better forms of therapy for these diseases.

Methods Employed:

Methods of protein purification and characterization, techniques of enzymology applied primarily to proteolytic enzymes and their inhibitors, kinetic analyses of enzyme reactions, procedures for quantitative measurement of various clotting factors, pharmacologic and physiologic techniques applied in man and animals, and assessment of metabolic pathways of blood cells with radioactively labeled substrates.

Major Findings:

1. Attempts to identify old and new platelets.

There are numerous reports in the literature suggesting that platelets change their size, specific gravity and metabolism as they age. Because ability to identify old and new platelets would be helpful in differentiating

between diseases of excess distruction and lack of production, we attempted to confirm these findings but were unable to do so on the basis of morphologic, physical, or biochemical characteristics. Specifically, by separating platelets into heavy and light populations we found that the two populations were of similar size and that their production of protein, lactate, CO_2 from C^{14}-labeled carbohydrate, and lipid from radioactive precursors were indistinguishable. Furthermore these two platelet populations separable by sedimentation reacted quantitively in the same way in aggregation phenomenon and in response to various agents that are known to agument or inhibit adenylcyclase and cyclic AMP levels. The two populations of platelets also survived the same period of time in vivo. These findings suggest that the age of platelets can not be discerned by current techniques. (Drs. N. Raphael Shulman, Richard Counts and Arthur Silvergleid).

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and should be helpful in defining new factors that accellerate or inhibit intravascular coagulation. (Drs. Richard Counts, Cecil Coleman and N. Raphael Shulman).

Honors and Awards: None

Publications:

1. Wolfe, S. M., and Shulman, N. R.: Inhibition of platelet energy production and release reaction by PGE_1, theophylline and cAMP. Biochem. & Biophy. Res. Comm. 41: No. 1, 1970.

2. Shulman, N. R.: Prothrombin consumption tests. In Bang, N. V., Beller, F. K., Deutsch, E. and Mammen, E. F. (Eds.): Thrombosis and Bleeding Disorders - Theory and Methods. London, Georg Thieme Verlag • Stuttgart, 1970, 79-82.

Serial No. NIAMD-CHB-2c
1. Clinical Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Study of the Immunology of Blood Cell Deficiencies

Previous Serial Number: Same

Principal Investigator: Dr. N. Raphael Shulman

Other Investigator: Dr. Richard Counts
Dr. Arthur J. Silvergleid

Cooperating Units: Dr. Lewellys F. Barker, LVI, DBS
Dr. J. Moor-Jankowski, New York University
Dr. Gerald Klatskin, Yale University

Man Years:

Total:	3.5
Professional:	2.5
Other:	1.0

Project Description:

Objectives:

To study the nature of immunologic reactions which result in formation of antibodies against autologous blood cells, and to determine the significance of immunity in development of "idiopathic" blood cell deficiency states. Of special interest are the biochemical reactions which result in formation of complexes between cells, antibodies, and drug haptenes and the physiologic processes which result in sequestration of cells with attached antibodies. In recent years our work on identifying inherited leukocyte and platelet isoantigens has led to a study of the significance of these antigens in hetero- and homo-transplantation and in possibilities of developing immunologic therapy against malignancies.

Methods Employed:

Techniques of quantitative immunochemistry, including preparation and physicochemical characterization of purified antibodies and antigens, micro-analyses for nitrogen, histamine, and alkaloid drugs, quantitative measurements of complement fixation, cellular agglutination and precipitin reactions, immuno-electrophoresis, methods of provoking antibody responses in man and animals, and isotopic and fluorescent labeling applied to antigens and antibodies. Methods of assessing reticuloendothelial function through clearance of labeled particles and organ localization of sequestered cells by external body counting. Tissue culture techniques, red cell agglutination techniques and lymphocyte transformation tests, including radioautography.

Major Findings:

1. A new test for viral hepatitis.

We developed a hemagglutination test for the antibody of long incubation hepatitis. This test is approximately one thousand times more sensitive than previous tests and as sensitive as the radioimmunoassay which was developed by others during the past year. Our hemagglutination test however is extremely simple, does not depend on any special reagent, and takes approximately 15 minutes rather than the several days radioimmunoassay takes to complete. With this test we have been able to detect antibodies in about 60% of patients following long incubation hepatitis. Antibodies frequently arise during the incubation period of the disease, and disappear at the time that viral antigen appears in the blood only to return later in the recovery period. Antibodies remain in the circulation for a matter of months or years thereafter but disappear in all but about 25% of cases after a year following acute hepatitis. With the new hemagglutination technique as many as 25% of normal blood donors have been found to have an anti-hepatitis antibody.

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Honors and Awards:

1. Patent for procedures involving complement fixation to detect hepatitis antigen and antibodies against it.

2. Patent for procedures involving hemagglutination to detect hepatitis antigen and antibodies against it.

Publications:

1. Shulman, N. R.: Hepatitis-Associated Antigen. Amer. Jour. Med. 49: 1970.

2. Bulkley, B. H, Heizer, W. D., Goldfinger, S. E., Isselbacher, K. J., and Shulman, N. R.: Chronic Active Hepatitis: Distinctions based on circulating hepatitis associated antigen. Lancet 2: 1970.

3. Vyas, G., and Shulman, N. R.: Hemagglutination assay for antigen and antibody associated with viral hepatitis. Science 170:332-333, 1970.

EXTRAMURAL PROGRAMS

ASSOCIATE DIRECTOR'S REPORT

Introduction

The highly organized, well run extramural operation recently has been under the guidance of Dr. Edward Offutt. The Extramural Program Directors are provided a matrix for uniform action in the handling of their research, training, and fellowship grant operations which maintains for them the individual options to respond individually and appropriately to the varying circumstances and problems that arise. The numbers of grants and the dollar amounts awarded by NIAMD are accomplished by a staff size which proportionately is much smaller than for any of the other Institutes. The Extramural Programs staff is very efficient; however, some aspects of the Extramural Programs performance have been necessarily compromised or sacrificed. The program directors administer their programs with various administrative-science mixes; the actions of some are skewed clearly toward the administrative side and of others toward the scientific side, either of which has both advantages and disadvantages. Overall, the best program administration which balances appropriately administrative and scientific concern is sacrificed somewhat because the large load per program forces the mixture away from the scientific end. Similarly, an overview of the administrative support mechanisms of NIAMD-EP reveals that past consolidation of major senior positions has necessarily sacrificed the depth of performance. Specifically, Mr. Linden Neff, as well as Dr. Edward Offutt, have carried multiple full-time responsibilities for the past few years.

The Extramural Programs have one major need, that of a more explicit rationalizing and definition of its research and training goals and activities; and analyses in depth of the end results of its individual programs. Although the EP has done considerable general analysis of its programs, limitation of numbers of staff has forced these efforts to be incomplete. During the past year attempts have been made to clarify what further work needs to be done in this direction and will be reviewed in the following section.

Areas of Concern

A major portion of the Associate Director's efforts during the past year has been directed toward the explicit definition of NIAMD-EP goals, activities, and estimates of accomplishment. Although much more detailed information is available, the major issues that have been discussed and evaluated by NIAMD-EP this past year will be presented in a brief skeletal fashion. These issues have been or are in the process of being explored by NIAMD-EP staff and to varying extent have involved discussions with the Institute Director, NAAMD Council, ECEA, and Office of the Director, NIH. Many of these items are interrelated. Together they indicate that the Extramural Programs staff has reviewed its present methods for administration of research, training, and fellowship funds and has in various ways explored whether or not present mechanisms for funding decisions are indeed the best methods. The staff has

discussed the reliability of priority estimates of scientific merit by initial review groups; it has explored the extent to which the present system is responsive to external categorical needs and demands; it has reviewed the categorical goals of the NIH as they relate to the non-categorical, non-specific support of excellence in general; it has attempted to study the influence of program planning and program emphasis in modulation of the scientific review process, both between Institutes and between programs within NIAMD; and it has asked how the program directors' functions might more effectively be pursued by modifications of responsibilities. These and other concerns are summarized in the following brief descriptions of major issues.

A. Quantitation of Research Activity in NIAMD-EP Categorical Program Areas

NIAMD-EP is organized in 11 program areas: arthritis, dermatology, diabetes, endocrinology, gastroenterology, hematology, metabolism, nutrition, orthopedics, urology and renal diseases, and physical biology and related areas. Each program area has funded the same percentage of the numbers of approved competing grant applications placed in each program; as a result the programs vary in size according to the numbers of grants approved within each program area. EP staff discussions have questioned the assumption implicit in this system that the level of research activity for a given program area resulting from a proportionate responsiveness to the demands (as indicated by the numbers of approved grant applications in each program area) is in fact the correct amount. There must be some important areas of research more inadequately supported than others!

Definition of support in each categorical program area is important because the program director tends to have a proprietary concern with those research efforts administered by him; similarly, the academic community in some large fraction is concerned with this localized and restricted categorical view of fundings for its areas of interest. Indeed, many categorical groups (e.g., the gastroenterologists) have expended much effort in defining the scope of the country's health problems related to their fields in terms of frequency of disease, disability, economic costs, death, etc.; and have attempted to measure the fraction of the total national resources supporting specific categorical research and training efforts against the scope of the health problem. Such groups fail to understand fully the extent to which there is crossover support between program areas within NIAMD, and indeed crossover with activities in other Institutes; on the other hand, we also do not fully understand the extent of this crossover because we have not seriously attempted to measure it.

An initial attempt was made to measure the crossover of research activity between the 11 program areas of NIAMD-EP using the available classification system. This system was originated for purposes of information retrieval (for responding to outside inquiries regarding specific research activities supported by AM Institute) and was not generated for purposes of describing the program as a whole. An attempt was made to use this retrieval system by determining the importance of the major descriptors to each of the 11 program directors, and by prorating dollars assigned to the various

descriptors used in that system. Although this analysis characterizing crossover did not provide a fully satisfactory answer because of its limitations, the results were in accord with the a priori belief that there was large crossover between program areas. However, it failed to provide convincing quantitation of the crossover. In part, the confounding of understanding of the crossover results from the fact that the program designations themselves are not homogeneous;e.g., diabetes is a disease, metabolism is a discipline, hematology focuses on a body system, etc. Because a major criticism of this analysis was the multi-disciplinary nature of the descriptors themselves, further analyses of the descriptors is in progress. However, it is likely that a new classification system needs to be generated if there is to be more precise definition of crossover between program areas and hence delineation of total support to any one categorical field. NIAMD-EP is interested in pursuing this in the future and NAAMD Council would be interested in obtaining such information. In the long run, such definition of research effort is necessary if NIAMD-EP is to further modify rationally its decision-making process as it influences shifting of research grant support between its various program areas. As of now, absence of a good reason to change leaves intact the NIAMD-EP principle of funding the same percentage of approved grants in any program area which in turn implies that this provides the best balance of research efforts. Further rigorous delineation of AM efforts in respective categorical areas is necessary to allow appropriate response to real categorical needs and demands from the external community.

However inconclusive the findings are at the moment, our efforts at such program analysis are most helpful in dealing with members of the scientific community interested in specific AM categorical areas, and releases what is otherwise argumentative leverage exerted by them in discussing the restricted amounts of money administratively assigned to any single categorical area.

B. Initial Review Group Priority Numbers as Indices of Scientific Merit

The priorities assigned by Study Section review as estimates of scientific merit constitute more than 90% of the total influence in the NIAMD decision-making process. Within AM Institute, staff and/or Council judgments of program relevance and other factors are modulating influences which modify the funding decisions between and within individual program areas. At a time when very good priority level applications are approved but unfunded the entire peer system is subjected to much criticism by some members of the external academic community. Because AM program areas interdigitate the priority scores stemming from many different Study Sections, and because the NIAMD principle of research grant funding allows differing priority cut-offs for the various program areas, the priority scores constitute the operational core of our funding decisions. For these reasons there is considerable need to know just how reliable such priority judgments are. Following staff discussion there has been presented to ECEA for discussion a rough proposal for a study which would allow the separation of variations in priority judgments resulting from differences between applications from those that are due to differences between review groups. Operationally, the present system assumes that variations in priority judgment differences

between review groups is zero, and hence all variation is attributed to differences in quality between the applications. This inappropriate assignment of the total variation to the applications themselves has been a necessary consequence of not having measures of variations between review groups. The essence of the proposal is that experiments be performed in which formal measures of judgmental variations between Study Sections is accomplished by routing a sample of research grant applications to multiple Study Sections for simultaneous review. Such an experiment necessarily would be limited to those research grant applications whose substantive content legitimately could be reviewed competently by more than one Study Section. Only by such an experiment could one find out that a given Study Section which assigns priorities which are on the average better than those of another Study Section has in fact better applications for review rather than that it is less rigorous in its standards. Within such a study one could also evaluate the effect of increased budget requests upon priority judgments, the influence of the time of day when reviewed by Study Section, etc. Whatever the results, a characterization of the degree to which such priority judgments are reproducible would be very helpful to those in funding decision roles; it would aid in determining whether programmatic modulating influences should be encouraged and expanded, or discouraged and minimized.

C. Objectives of NIAMD Categorical Training Programs

Against a background of a large NIH-wide study of its training programs, NIAMD-EP staff has spent considerable time in discussing its own categorical training grant programs. Within its categorical areas of responsibility NIAMD has provided training in very large part in clinical departments for the development of researchers and faculty members. This is seen as a critical and necessary part of the spectrum of activity leading from discovery of new knowledge through development and innovation to application and translation of that new knowledge into ever improving quality of patient care. NIAMD-EP has initiated some and is planning other studies to describe and link the full chain of events that does in fact show progression from the origin of new information to improved quality of patient care. It plans to do this within the limits of the categorical areas of its responsibility. Hopefully information so generated will be valuable to the NIH operation as a whole.

NIH training programs complement residency programs in medical institutions across the country so as to provide the whole needed spectrum of the various mixes of research, teaching, and patient care careers. It is a widely held conviction that patient care is superior in those institutions that have significant amounts of training (and research). Attempts will be made to more explicitly define the quality and quantity of the end products of NIAMD training programs; and to link these end-products to estimates of faculty need for research and for teaching specialty subjects to medical students, house staff, and other health professionals who will fit into the health care delivery system of the future. It is believed possible to integrate studies of the appraisal of quality care with studies of NIAMD support mechanisms to confirm or deny the commonly held belief that such training

programs contribute significantly to the quality of training provided medical students and young physicians and which result directly in improved quality of patient care service. Such studies limited to diseases of categorical interest to NIAMD constitute a major portion of internal medicine and perhaps may be of some use by extrapolation to the NIH as a whole. Protocols are in early stages of preparation to be submitted for evaluation and review which would study selected disease areas of importance to and responsibility of NIAMD. In addition, because of the interdependence of quality of care available for any one categorical area with the many others in the same institution, it may be necessary to evaluate the differences in overall quality of medical care between whole institutions to be able to analyze the direct effect of the presence of NIH training and research support.

NIAMD staff and Council have reviewed many factors which might influence operational decisions regarding NIAMD training grant funding. They include
(a) avoidance of abrupt departures from past patterns
(b) evaluation of academic faculty productivity as related to need
(c) amount of training support from other sources
(d) total amount of research support available
(e) the presence of a minimal critical mass for training
(f) estimates of needs for researchers, faculty, and practitioners (which in turn relates to the scope of disease in terms of frequency, disability, economic loss, death, etc.)

Discussion of these factors has led to attempts to define explicitly items which are used to modulate priority judgments and which potentially can lead to shifting of support between the various program areas within NIAMD-EP.

D. New Training Instruments in Digestive Disease and Nutrition

Review of NIAMD training and fellowship support by staff led to definition of two areas for which new training instruments could provide important support. The first was designed to deal with the gap between early demonstration of potential as a research trainee and eventual qualification as an investigator or faculty member. This instrument ("Clinical Investigator Award") is for three years and is designed to meet specifically the needs for more research development. The second instrument ("Academic Career Development Award") would allow faculty development with great emphasis on teaching and patient care but with some meaningful research component. It is for five years and offers support to a faculty member who would aid in the development of a specified categorical program within an institution. Both of these instruments are being implemented only in the fields of digestive disease and nutrition, categorical areas with large national shortages. Of importance is that the NIAMD-EP has sought to versatilize the NIH training instruments available to aid in the development of strong faculty members in adequate numbers to deal with the needs of health professional institutions in its areas of responsibility.

E. Location of Scientific Decision Making Process for a Categorical Area

The Extramural Programs staff has had extensive discussions regarding the best way to have grant funds support excellent and diverse activities in the

many categorical research areas for which it has responsibility, while maintaining the principle of supporting those efforts which on the basis of the best available scientific judgment appear to offer the greatest opportunities for gaining new, meaningful, and useful knowledge. Views range between two extremes. At one end is the view that the EP should function in an almost completely passive fashion; that it should receive grant applications, process them, and fund them solely on the basis of the priority judgments of scientific merit provided by initial review groups. At the other extreme is the opinion that categorical needs, desires, and demands should exert a major modulating effect upon the scientific merit judgments. This latter view suggests that the frequency, severity, mortality, and economic importance of disease processes constitute major facets in the overall decision making of the Institute.

Modulating the scientific merit judgments also could include concern for establishing a well balanced complementary distribution of NIH research investments from the view of the country as a whole. This would require extensive study and analysis of all research supported by other Federal agencies, other Institutes, and by industry and foundations. Analysis of such other support would be needed before the Institute could exert a complementary decision making role, which until the present the Institute has done in an incomplete and indirect fashion. Were this to be done, the EP staff would delineate the character of research being supported by all other Federal agencies, private industry, and other private sources as a preliminary step; present this information to the Council; and provide Council with the option of using such information to modulate the advice it gives, thereby allowing a greater role for program importance, relevance, etc.

Many of staff and most of the Council favor the view that categorical ends would best be served by support of excellence, per se, whatever its momentary categorical nature, and that to skew deliberately distribution of funds for seeking greater gains in specific categorical areas may in fact reduce total gains to that as well as to all other categorical areas. Despite the Council's reaffirmation of the principle that it wished not to deviate much from the principle of supporting those projects with greatest scientific merit as judged by peers, there remained evident sympathy for individual categorical needs (e.g., by its decision to continue funding a similar percentage and hence a different number of approved grants within each program area). Much more discussion would be necessary before the Extramural Programs could in fact move toward providing information that would allow program factors to increase as modulating influences upon judgments of scientific merit in the funding process.

Additional investigations are required to test the hypothesis that our current system undergirds, namely, that the nature of the distribution of research effort seen within the applications coming from the field represents the best pattern for research support. The possibility of areas of research which are "over-saturated" or for gaps in research effort should be studied by external advisory groups and methods explored for dealing with these within the context of our general operational principles.

A more active role of Extramural Programs staff in relation to categorical areas of responsibility would require considerable increase in the staffing pattern to support generation of the underlying information required. As a by-product of discussions we have explored in an initial fashion the potential advantages (and disadvantages) to establishing a small contract arm in liaison with each of our grant program areas.

F. Reorganization of Digestive Disease and Nutrition Programs (DD-N)

A recent reorganization of NIAMD in regard to its digestive disease and nutrition programs has brought into focus many of the issues discussed in Sections A through E above. Reviews by external categorical groups of gastroenterologists and nutritionists of their fields has suggested that there is a discrepancy between the scope of those categorical diseases as national health problems as compared with the support provided by NIH in these areas. It is our staff's view that it is very difficult to measure precisely the amounts of support to individual categorical areas. Indeed, in Section A, above, it was indicated that a great deal of crossover exists between our program areas so that research of importance to gastroenterology and nutrition is much greater than that which is administratively handled within those two programs. Nonetheless, there is a general sense that, although very difficult to measure quantitatively, there indeed is a great deficiency in support in these two areas as compared with their importance. Thus, in an attempt to be responsive to external demands and legitimate needs, the fields of digestive disease and nutrition are to be given greater visibility and support through a series of organizational changes centering about the appointment of an Assistant Director for Digestive Disease and Nutrition. These two programs will be combined under the Assistant Director and will be provided an opportunity to exert independent and autonomous modulation of scientific merit in their funding decisions for research, training, and fellowship programs. This will allow a localized test of our ability to demonstrate an increased programmatic responsiveness to the needs of the categorical fields while at the same time attempting to maintain the general operational principle supporting scientific excellence primarily. It should provide possibilities for greater program identity and more innovation in reaching goals important in these categorical areas.

Operationally, a major change will be the isolation of proportionate amounts of the current research, training, and fellowship budgets for use by the Digestive Disease and Nutrition Programs independent of the funding of those instruments in the remaining nine program areas. The possibility of innovation is indicated by the recent proposal for two new training instruments in Digestive Disease and Nutrition, referred to in a discussion of training grants, Section D above. These two new instruments, the Clinical Investigator Award in DD-N and the Academic Career Development Award in DD-N, represent examples of the shifting of fund utilization toward activities which are believed to be more effective in the development of research and teaching faculty.

With an isolated and autonomous administrative status, the priority cutoff within the Digestive Disease and Nutrition research program areas may deviate from other programs. It is the sense of the Extramural Programs staff that

this autonomy will not be used to support poor quality research; rather, it will provide the flexibility for establishing new instruments (e.g., centers for study of specific diseases) and other approaches to improve total research productivity. The Assistant Director for Digestive Disease and Nutrition will be substantively independent and will report directly to the Director, NIAMD. He will collaborate with the Associate Director for Extramural Programs for operational support of DD-N grants.

The Assistant Director for DD-N will develop a contract mechanism to complement the grant activities relying on advice provided by outside specialty groups as to areas which need to be pursued which are not being adequately explored via the grant mechanism. Contracts can be initiated to aid the overall progress of both research and training efforts in DD-N. Operationally, the Assistant Director's contract efforts will be coordinated with the existing NIAMD contract operation.

The Assistant Director for DD-N will have responsibility for establishing a clearing house of information and a coordination effort for all of NIH regarding digestive disease and nutrition. A product of this activity will be a more explicit and documented characterization of the scope of the digestive disease and nutrition problems, the total amounts of support from all sources directed toward these areas, and dissemination of information to the public. This information can be invaluable in providing the basis for request of appropriate increases in research, training, fellowship, and contract support in the future.

Administrative Structure

One year's experience in NIAMD-EP has revealed a highly organized, well integrated, and efficiently run organization. Its Operations, Grants Management, and Analysis sections perform their functions accurately and efficiently despite an extremely large load for the number of staff. As previously stated, Dr. Edward Offutt and Mr. Linden Neff have occupied multiple full-time positions. A minimal alleviation of the load of Dr. Offutt has been provided by the appointment of Dr. Wilford Nusser as Acting Scientific Programs Branch Chief, one of the several positions Dr. Offutt had previously been carrying. Dr. Nusser has reorganized and supervised the program directors and their grants assistants to increase interplay between programs in their support of each other administratively. The appointment of a Scientific Programs Branch Chief on an acting basis was taken as an interim step in the face of other potential organizational changes of NIAMD-EP in the near future. The individual program directors have had increased demands placed upon them in the direction of increased characterization, analysis, and evaluation of their research, training, and fellowship grant programs. It is apparent that if significant escalation of such efforts is to be continued, additional staff for the Extramural Programs will be required.

Dr. Joseph Michalski continues to function as the Research Grants Officer, maintains excellent liaison with DRG, remains as a source of information regarding research grant policy and procedure, and serves as a focal point for liaison with other NIH components.

The extensive administrative and evaluation activities of Dr. William Batchelor are summarized in his report as the Training and Fellowships Officer.

NIAMD-EP Budget

It is the general consensus of EP staff that budgetary restrictions have sacrificed much good research in all of our categorical program areas. The percent of approved applications which are funded has ranged between 35 and 40 percent at time of Council with some additional applications funded from savings during the course of the year. During FY '71, research grants have been negotiated at the level of 10% on the average so as to provide additional funds for competing applications. We are following a plan to reduce the level of such negotiations during '72 to approximately half that of the present fiscal year, and intend to fully abandon this practice by FY '73. Similarly, in the training grant area we have negotiated reductions of continuing applications to provide funds with which we have minimized the reduction in number of ongoing training grants. It is believed that the temporary expedient of such negotiations for the past two years has indeed improved the overall status of our categorical research and training programs.

In an attempt to evaluate the effect of reductions in available research grant funds, we are initiating a study in which those grant applications which were approved but had priorities just below that which would allow payment will be evaluated to see the impact that might have occurred had even slightly greater amounts of funds been available.

The Extramural Programs staff also has been concerned about the influence of the progressively increasing disparity between NIH stipend levels and those of house staff salaries of physicians in chronologically similar stages of training. We are evaluating many claims that the differential in these levels is threatening extinction of many important training programs of AM Institute by virtue of lack of trainee applicants.

Summary and Future Directions

A major effort needs to be established for more detailed analysis and evaluation of the Extramural Programs as they relate to NIH and HEW missions. It is believed possible that the appropriate support of research and training as an integral and crucial part of an overall health care system can and should be documented in the usual terms of effectiveness and efficiency of expenditures. During the past year initial efforts have been made in these directions of analysis. Such efforts have been possible only because of the remarkable efficiency of the general operation of the Extramural Programs. Nonetheless, these efforts have been necessarily limited by time and personnel constraints, and it is believed that additional staff is a requisite if the kinds of studies described above are to be done in the near future with the degree of thoroughness and reliability that is needed.

Special Earmarked Arthritis Research Funds

Applications received, reviewed, and funded for five years with the "Special Earmark Funds" from FY 1967 will be going into the final year of the approved project period beginning June 1, 1971. It would seem appropriate to provide a brief interim review of this program. Sixteen applications were approved under this special program, of which two were withdrawn and fourteen funded. Of these, nine were approved for five years, three for three years, and two for two years. Only one of those funded for less than five years has successfully competed for funding when subsequently reviewed by a regular study section.

Research proposed in the nine applications entering the final year of the approved project period include studies of drug therapy (in 8), synovectomies (in 2), epidemiology (2), SLE (3), etiology (2), differential diagnosis and new diagnostic criteria (2), immunology (3), psychosocial studies (1) and genetic studies (1). Support for an additional eleven ongoing research projects was transferred to these "special funds," of which seven are no longer active.

A short summary of one of the two epidemiology studies is reported below. A detailed study was made of 176 patients divided into groups of rheumatoid arthritis (53), possible variants of rheumatoid arthritis (46), and other categories of arthritis (77). Preliminary evaluation of these patients produced the following observations:

1. No age, race, or sex differences were found between RA and the variants of RA.

2. Data indicate that the RA and RA variant have similar characteristics at onset of the disease and may belong to a common clinical syndrome whether or not their etiology or prognosis are similar.

3. The RA patient had a significantly less frequent history of allergies.

4. The average number of cardiac symptoms was significantly more frequent among RA cases than controls.

5. RA patients had a consistently lower average 17 Ketosteroid excretion than controls.

Continuing studies of this type will help to elucidate and define differences between rheumatoid arthritis, variants of rheumatoid arthritis, and similar or related diseases. A survey of the incidence of RA in native Puerto Ricans is nearing completion and results of this study should be available in the near future.

Immunologic Research

Requests for research support in immunology are increasing, with special emphasis on the immunoglobulins and their possible relationship to arthritis as a causative factor, and on the various changes that are observed in the immunoglobulins concurrent with this disease. Recent developments in immunology that may be of major importance are:

1. The complete amino acid sequencing of a human $\gamma G1$ immunoglobulin (Eu) has been determined.

2. The first genetic marker of serum IgA, a major immunoglobulin of exocrine secretions, has been defined and termed AM(1). It is localized in the α-chains of the γA_2 subclass and is independent of the serum γA_2 level.

3. Four of the six fragments produced from the heavy chain of IgM by CnBr cleavage have been obtained in pure form.

4. Human antiglobulins to IgA have been found. Some react with all human IgA, others with genetically determined antigens in IgA.

5. Isolation of a connective tissue activating factor (peptide) which can be extracted from various sources, including leucocytes and synovial cells. This material stimulates glucose utilization, lactose production, and hyaluronate synthesis, properties which distinguish cells of inflamed vs. normal synovium in tissue culture.

Microbial Research

Studies of microbial relationships to RA are continuing. Mycoplasma closely related to human forms have recently been isolated from subhuman primates. Studies will be undertaken to determine if "arthritis-like" conditions occur in primates following injection of these organisms.

One five-year long-term study of "slow virus" as an etiological agent of arthritis in subhuman primates is entering its fourth year and has one additional year to go before the animals will be sacrificed for appropriate post-mortem studies. A second long-term study at a more sophisticated level has recently been initiated.

Synovial cells from human arthritic donors, grown in tissue culture, have been shown to resist penetration by Newcastle Disease virus and measle viruses injected into the culture media. This observation of virus exclusion may indicate the presence of a virus or virus-like particle in synovial cells. However, additional studies will be necessary to fully evaluate these preliminary findings and to determine if this is truly a virus exclusion phenomenon.

Other Studies

A grantee has been able to partially extract the Z line of muscles from I-Z-I brushes. Various extraction procedures remove much of the muscle

protein without disturbing the Z line. The final steps involve the removal of myosin, and then the Z disks by repeated gentle washing. Initial studies indicate that actinin is closely bound to or may be the major constituent of these disks. Biochemical and histochemical testing of the isolated Z disks should provide fairly reliable data on the molecular composition of this part of the muscle fiber.

Research on collagen and scar formation has led to the discovery of a potential model for studies of cirrhosis in humans. Dogs injected with dimethylnitrosamine (DMN) intermittently for four weeks developed hepatic cirrhosis which was stable or progressive for at least five months after discontinuation of the drug.

02 - DERMATOLOGY PROGRAM AREA

As in the recent past, NIAMD-supported projects administered through the Dermatology Program area reflect an increasing diversification of studies extending beyond dermatology per se to diseases, experiments, and experimental models where the integument of animals and human beings is involved.

Transplantation studies currently underway involve the production of graft tolerance with non-viable substances. Efforts are being directed toward the isolation and characterization of transplantation antigen from the guinea pig and its ability to induce immune tolerance to skin grafts. The bioassay of isolated fractions utilizes the development of a delayed hypersensitivity reaction in inbred guinea pig strains.

Using isolated frog skin ion transport across biological membranes is being studied. Sodium transport is a major function of living cell membranes but little is known about its specific molecular nature. The nature of the metabolic linkage is also obscure. Utilizing the isolated frog skin opens the possibility of measuring sodium fluxes across the individual faces of the epithelial cell.

Photobiology is a rapidly emerging new research discipline. Investigators are continuing a study of the photochemical, biochemical, and biophysical alterations which are responsible for the development of erythema following exposure to ultraviolet light. Previous work supported by AM grants has shown that urocanic acid is present in epidermis in sufficient quantities to absorb an appreciable amount of ultraviolet light. It has been further demonstrated that photochemical transformations of urocanic acid occur in vivo. There is currently an attempt to identify enzymes in skin which show increased activity after exposure to ultraviolet light. This is an important field of research in terms of environmental effects upon human skin and it is hoped that additional studies will unify the multiple observations and findings being reported into a coherent concept of the role and significance of the biochemical reactions in radiation damaged tissue.

A young investigator has reported that carotenoid pigments protect bacteria and animals from photosensitization and apparently also protect patients with erythropoietic protoporphyria. There is great biologic importance of carotenoids in photoprotection from both clinical and basic biologic viewpoints.

Elucidation of eccrine sweat gland function is important not only in relation to developing better therapeutic agents for the treatment of heat intolerance and other sweat gland-related diseases but also has potential significance in learning more about the disease process in cystic fibrosis where children afflicted by this disease demonstrate sweat gland abnormalities. In the initial period of one grantee's research, sweat gland function had to be determined by the fairly crude method of collecting sweat from relatively large areas of the skin surface. Although this technique has yielded much useful information, he is now able to perform his studies on isolated whole sweat glands or on the secretory and ductal portions separately. The use

of the cat's paw sweat gland for in vivo studies is also a new development. In this animal the sweat duct acts only as a passive conduit and allows alterations in the formation of secretory fluid to be determined easily. As a result of these improvements in technique, more information about the mechanisms for controlling water and electrolyte transport in the eccrine secretory coil and duct should be available in the near future.

Keratohyalin granules are thought to play an important role in the maturation of skin. One attempt to understand the pathways of keratin synthesis involves the study of alterations in epidermal and hair root cell-free protein synthesis in response to exogenous agents. A grantee is studying the clinical patterns in more than a hundred and twenty patients who suffer from various disorders of keratinization. Concerted efforts will be centered on applying techniques developed for the study of keratin from genetically determined disorders of keratinization to determine the specific defects in these patients. An area of great importance is evolving as a result of these grant-supported studies concerning the keratinization process; it would appear that the control of epidermal developments may come about through the control of mitosis. At present there are strong suggestions that accelerators and inhibitors are operating, the latter suggested by the action of the so called "chalone" systems. Serious consideration will be given to new applications concerning the control of mitosis as such new information may relate to finding a control for psoriasis and other keratinizing disorders.

As a result of greater efforts being made to understand the pathogenesis of psoriasis the NIAMD will sponsor a Workshop at the NIH in October 1971 to review those research areas that are most relevant to further progress in the search for a cure or safe control of a disease that affects an estimated six million Americans. This hyperproliferative disease is similar to cancer in that there is loss of cell control. Research in areas to control mitosis therefore may have a significant bearing on current cancer studies. It is hoped that the Workshop will more clearly define what areas must be pursued in psoriasis research.

The hormonal control of sebaceous gland function is studied in the search for some agent or agents that will control acne vulgaris before it results in the physical and emotional scarring of adolescents and young adults. It has been clearly demonstrated by grantees that patients with acne in whom sebum production was significantly reduced by estrogen administration showed clinical improvements. However, the estrogens can be administered only to females as there are feminizing effects when administered to males. To circumvent this there have been attempts to develop anti-androgens. These anti-androgens have been studied recently for their effect on sebum production by topical application to the forehead of normal male subjects. Unfortunately no reduction of sebaceous secretion has been observed. The role of micro-organisms is also being evaluated as one of the pathogenic factors in acne vulgaris. It has been determined that any therapeutic agent which will either decrease the amount of sebum that is produced or prevent the hydrolysis of fatty acids in sebum will be of aid in the disease. There is a direct correlation between the C. acnes population and the level of free fatty acid levels in the skin. The administration of tetracycline reduces

the free fatty acid levels in subjects with high C. acnes population but does not produce any change in the free fatty acid concentration in subjects lacking a significant C. acnes population initially. These studies indicate the probable importance of C. acnes as a source of lipolytic activity. While the various sulfonamides have been ineffective in altering the composition of sebum, the combination of sulfisoxazole with the potentiator trimethoprim decreases the free fatty acids in sebum; thus there is a possibility of using an antibacterial agent that has a different mode of action than the tetracyclines. The importance of these acne studies is highlighted by the report that the amount of money spent on over-the-counter acne aids in 1966 amounted to thirty-seven million nine hundred thousand dollars. This figure obviously has increased; thus the cost of acne care is very great indeed.

Another research effort with broad implications is an investigation of the origin of lipids accumulating in xanthomas. It is hoped that the cutaneous lesions might serve as a useful model from which to draw significant analogies relative to the atherosclerotic process. Observations made to date reveal that while plasma-borne lipids significantly contribute to the lipids found in xanthomas this is not simply the result of passive deposition of lipoproteins; rather, the kinds and amounts of lipids found in xanthomas is a result of a series of reactions involving deposition of lipoproteins from the plasma, mobilization of certain of the lipids back into the blood stream, local in situ synthesis and probably transesterification of various accumulating lipids.

Immunofluorescent studies were wholly investigational techniques at one time, but now are becoming almost routine diagnostic services. Immunofluoresence has made it possible to gain enough insight into the immunopathology of pemphigus to render it now one of the best documented examples of an autoimmune disease caused by circulating antibodies.

Relatively little is known about the organization, growth and differentiation of skin. One grantee is investigating the epidermal-dermal interactions in normal and diseased adult human skin, particularly with reference to the role of dermis in the maintenance and control of epidermal organization. In recent studies dermis was found to be essential for the maintenance of adult epidermal characteristics. Evidence has been provided for the presence of a soluble mediator of epidermal maintenance produced by dermal fibroblasts. Although the specific soluble factor responsible for this effect has not been identified the ultimate identification of this factor responsible for epidermal maintenance would be of great importance to the understanding of epidermal-dermal interactions in both normal and diseased human skin.

In studies of wound healing it has been observed that both mitotic and differentiating cells participate in formation of a protective cover over skin wounds. The cells which migrate from the epidermis divide and differentiate while they move over the wound surface. Thus mitosis and differentiation are not incompatible with migration as has been postulated by previous workers.

The role of human skin collagenase in the degradation of collagen in normal and diseased human skin as well as the relationship of this enzyme to other human and animal collagenases is currently under investigation. Greater knowledge in the area of collagen synthesis and degradation may help in understanding the enigmas of scleroderma, dermatomyositis, and other diseases

that have strong dermal components.

Evaluation and use of frozen storage of skin grafts is currently under investigation. Some of the clinical studies associated with this project studying the relationship between oxygen utilization and tissue viability have been productive of data concerning the relationship of skin graft acceptance to the bacterial flora of chronic wounds of many types.

Effects of prostaglandins on developing skin havebeen reported to show a specific alteration in morphology of mitochondria. The significance of this work is three-fold: (1) determination of the factors responsible for the alteration in mitochondrial morphology would aid greatly in establishing a mechanism of action for the prostaglandins, some of which hold great promise for medical applications, (2) the above might shed new light on the so called "inductive" mechanism during organogenesis, and (3) the size reduction of treated explants may be an excellent model for the phenomenon of clot or graft retraction and may serve adequately to test the present concept of fibroblast contractility.

The major aims of the diabetes program are to define the disease in terms of its causes and the many complications which are associated with it, and to find methods of prevention. To prevent or control diabetes, two goals must be achieved. First, better methods must be established for early detection and prediction of the disease. Second, the fundamental genetic and metabolic causes of the disease must be fully understood so that rational methods of prevention and treatment can replace the present empirical and inadequate approaches.

During FY 1971, the support of approved, on-going projects was reduced by the limited availability of funds from 135 to 118 grants. All of the 100 continuation projects received an average of 10% less than the recommended funds, which is expected to reduce their productivity. Only 18 of the 35 approved renewal applications could be funded. Of 31 approved new proposals only eight will be funded. The 23 new proposals which cannot be supported include many excellent projects.

A decrease in training activities in diabetes is also in prospect. Of 21 training grants active during the current year, only 18 will be supported starting July 1, 1971. Only one competing renewal and none of three approved new applications will be funded in the next year. A similar decrease in the number of training grants is predicted for the next year. Of the 12 research career development awards (RCDA) active in fiscal year 1970, 10 will remain active during fiscal year 1972. Only one new RCDA was initiated during the current year. Eighteen fellowships related to diabetes were active at the beginning of the current year, with no appreciable change expected. The program continues to support one research career award. The overall decrease in these training activities is regrettable, because they constitute important sources for the instructors needed for staffing new medical schools and for increasing the number of physicians trained in existing schools.

The 126 research grants constitute the most direct approach to the many problems associated with diabetes; the training activities supplement these reseach efforts. A wide range of objectives, approaches and interests are reflected in the research projects; these have been classified for planning purposes into four major groups described below.

Etiology, Pathogenesis and Treatment.

Fifty-six of the 126 projects are concerned with the causes and diagnosis of diabetes, the complications of the disease, and the efficacy of current or potential treatments. These projects place great emphasis on the study of diabetes in human subjects to complement research in experimental animals. Included are studies of the role of inheritance and diet, changes in metabolism and enzyme concentrations in the diabetic state, characterization of the complications which follow initial development of the disease, study of the mechanism by which known diabetogenic agents cause or simulate the disease, and testing of current and potentially new methods of treatment. Only one or two projects involve detailed studies of the development of diabetic vascular complications, and much more emphasis on this subject is needed.

Nineteen of the 56 projects are designed to determine the extent to which hypoglycemic agents (insulin and synthetic drugs), or carefully selected diets, control the complications developed in the diabetic state (including diabetic retinopathy and neuropathy, glomerulosclerosis, microangiopathy), and effects on longevity.

A major report on 13 of the projects, which constitute a collaborative effort known as the University Group Diabetes Program (UGDP), was presented and published during the current year. The Group concluded that a combination of diet and therapy with tolbutamide (a widely used oral hypoglycemic agent) is no more effective than diet alone in prolonging the life of diabetic patients. Moreover, the findings suggest that tolbutamide and diet may be less effective than diet alone or than diet and insulin at least insofar as cardiovascular mortality is concerned. For the latter reason, the use of tolbutamide was discontinued by the Group. It was stated that the conclusions mentioned above pertain only to the type of patients studied and to the dosage schedule used by the Group. Additional observations are needed to determine whether or not the results of the UGDP apply to diabetic patients generally. There is little doubt that the treatment of many diabetics has already been changed as a result of the UGDP report.

Four approved applications for renewed support and 12 for new projects, classified in this group, could not be funded.

Secretion and Activity of Insulin and Related Hormones.

Thirty-six of the 126 projects involve studies related to insulin secretion and activity under a variety of circumstances in normal and diabetic patients and in experimental animals. This includes the histology and physiology of the pancreas, insulin biosynthesis in isolated cells and in secretion granules of the islets of Langerhans and the many factors regulating this synthesis, abnormalities in insulin secretion in the diabetic patient or animal, improved techniques for measuring insulin activity, antibodies and inhibitors which may block insulin action, factors which affect the degradation of insulin, and other hormones which may oppose or supplement the action of insulin.

An exciting development is the finding that diabetes is characterized by a continuous state of relative or absolute excess of glucagon, a hormone which is antagonistic to several actions of insulin. Therefore, the diabetic may suffer from both a lack of insulin and an excess of glucagon. While there are many questions to be explored in connection with this new finding, the implications are important, and it is entirely possible that improved treatment of some types of diabetes will develop from this observation.

Seven approved applications for renewed support and 8 for new projects, classified in this group, could not be funded.

Mechanism of Action of Insulin.

Twenty-five of the 126 projects are primarily concerned with the precise biological functions of insulin and the mechanisms by which these functions are fulfilled. Subjects under study include the relationship of the structure of insulin to its activity, factors which modify insulin action, the role of other hormones in regulating insulin activity, the mechanism by which insulin controls blood sugar levels, and the effects of insulin on the metabolism and transport of carbohydrates, lipids and proteins. These studies are fundamental in nature and commonly involve investigation at the molecular level.

A new project initiated in this area during the year is based on the proposition that insulin may function by activating specific genes, thus permitting genetic expression leading to the synthesis of certain proteins including enzymes. While this is a difficult problem in a technological sense, it is important that this proposition be investigated. If activation of one or more genes is essential to the normal action of insulin, and if the exact loci can be determined, it may aid in development of drugs which would simulate the action of insulin in the diabetic patient.

The single research career development award initiated in the program during the year will permit application of affinity chromatography, by an exceptionally gifted individual, to a study of the roles of insulin and glucagon in regulating the translocation of sugars across cell membranes.

One approved application for renewed support and three for new projects, classified in this important group, could not be funded.

Carbohydrate and Lipid Metabolism.

Nine of the 126 projects are best characterized as comparisons of carbohydrate and lipid metabolism in diabetic vs normal individuals or animals. Subjects under investigation include pathways for catabolism of carbohydrates and lipids, gluconeogenesis, synthesis and interconversions of fatty acids and lipids, storage and mobilization of lipids in adipose tissues, and the control of such processes by hormones, particularly insulin. Such studies are, of course, essential for a full understanding of the changes which occur in the diabetic state.

Five approved applications for renewed support, classified in this group, could not be funded.

04 - ENDOCRINOLOGY PROGRAM AREA

Science quietly advances mainly by the fitting together of small bits and pieces of information. Occasionally a larger step occurs that deserves more attention. In January 1971 Dr. C.H. Li reported the synthesis of human growth hormone. This important synthesis eventually should enable medical specialists to treat pituitary dwarfism easily and inexpensively. Dr. Li has been a NIAMD grantee for 17 years, working on the purification, structure, and synthesis of lactogenic and gonadtropic hormones, other hormones produced in the pituitary gland. Other important work across the country has been supported by NIAMD leading to increased knowledge of hormone structure and endocrine function in health and disease.

A rapid method for disseminating scientific results and stimulating new research is by conferences at which specialists and interested scientists present their latest findings and discuss future work. This past year two such conferences were enthusiastically received by the scientific community. In January a Prolactin Workshop was held at the NIH, sponsored jointly by the Endocrinology Study Section and the Reproductive Biology Study Section. Topics discussed included chemistry, immunochemistry, assay methods, physiology and pathology of prolactin. In March, the University of North Carolina was host to the Fourth International Parathyroid Congress, sponsored in part by NIAMD. All phases of calcium metabolism and its hormonal control were considered during the 5-day meeting.

The following contains some highlights of the past year in endocrinology research:

Pituitary Hormones

In addition to the synthesis of human growth hormone, other studies give insight into the mechanisms of action of the hormone. Kostyo reports that growth hormone stimulates protein synthesis in rat diaphragm muscle, but only after a lag time of at least 20 minutes. He believes that it may work by activation of an enzyme system within the cell, the lag being due to metabolic processes which must first occur. Additional evidence is accruing that only a portion of the growth hormone molecule is necessary for its anabolic effects. Using a radioimmunoassay technique it has been shown that the physiologic secretion level of growth hormone in humans may be determined from blood samples taken after a period of sleep.

Two studies of growth hormone relate to acromegaly, a disease of postpuberty in which the hormone stimulates continued growth of hands, feet and lower jaw. In patients with Parkinson's disease treatment with the amino acid L-dopa unfortunately appears to cause increased growth hormone production, thus causing acromegaly as a side effect. On the other hand, NIAMD grantees reported that a synthetic progesterone, medroxy progesterone acetate, would decrease growth hormone release, thus alleviating symptoms of acromegaly.

Animal studies over the years have confirmed that there are six anterior

pituitary hormones. Until this year, there was some doubt as to whether human growth hormone and human prolactin were separate entities. Two reports from NIAMD grantees now show that the hormones are separate in humans as well as in lower animals. Daughaday reports prolactin activity from human pituitary extracts which is separate and distinct from human growth hormones. Frantz and Kleinberg report that a sensitive bioassay has enabled them to separate GH and prolactin in the plasma of human sujbects.

Dr. C.H. Li has succeeded in determining the complete amino acid sequence of ovine prolactin. Comparison of this structure with that of human growth hormone shows a close similarity of the two hormones at one end of the chain, thus perhaps showing the reason for lactogenic activity of HGH. The structure of ovine prolactin has also been reported independently by Teh H. Lee.

Thyrotropic hormone from the anterior pituitary is released under the influence of thyrotropic releasing factor from the hypothalamus. In last year's report, it was noted that TRF had been isolated, and characterized as a tripeptide. Now synthetic TRF has been injected into human volunteers and its effect on TSH production studied. In normal subjects TSH was released when TRF was administered, whereas in subjects with pituitary dysfunction, the TSH level did not go above baseline levels. As an illustration of the complex interactions between endocrine glands, one report noted that diurnal variation of thyroid hormone release was due to variations in thyroid stimulating hormone from the pituitary, as would be expected. However, the variation in TSH appeared to be caused by variations in the amount of hydrocortisone from the adrenal gland, increased hydrocortisone causing decreased TSH production.

Thyroid Gland

The mechanism of action of thyroid hormone is unknown. Total body metabolism increases under the influence of this hormone, but the specific cellular effects are unknown. Work from several laboratories continues to explore the relationship of thyroid activity to specific cellular events. Studies of LATS (long acting thyroid stimulator) show that there is no apparent correlation between circulating LATS levels and thyroid function parameters in the hyperthyroidism of Graves' Disease. Furthermore, thyroid non-suppressibility in Graves' Disease may reflect an intrinsic thyroid abnormality, rather than the presence of circulating LATS.

Parathyroid and Calcitonin

It has been shown that calcitonin derived from salmon has a greater effect than that found in mammalian species in preventing bone destruction. The reason appears to be due to a difference in structure of the salmon calcitonin, which has differences in 11 of the 32 amino acid positions found in other species of calcitonin. This finding has led to the postulate that calcitonin may be more important in lower vertebrate forms, especially fish, than in man, and it may in fact be considered as a hormonal vestige in man.

Two investigators separately but nearly simultaneously reported on the

structure and partial synthesis of parathyroid hormone. This hormone has a chain of 84 amino acids. Furthermore, a portion of this chain containing 34 amino acid residues appear to have all the biologic activity of the full chain.

Steroid Hormones

Steroids are produced by the adrenal glands and by the gonads. Although most studies on gonadal physiology are supported by NICHD, basic studies on sex steroids are supported by the Endocrinology program of this Institute.

NIAMD scientists have described the role calcium plays in ACTH stimulation of the adrenal gland. Production of adrenal steroids occurs when the adrenal is stimulated by ACTH from the pituitary. Several steps occur. (1) ACTH is bound to a receptor. (2) The enzyme adenyl cyclase is activated by ACTH, which (3) converts ATP to cyclic AMP, and (4) cholesterol is converted to corticosteroid. Calcium is necessary for the ACTH activity.

The ACTH receptors referred to above, have been used in a new assay procedure. Adrenal ACTH receptors were extracted and bound by radioactively labeled ACTH. When exposed to unlabeled ACTH in plasma, the labeled ACTH was displaced by unlabeled hormone. By determining the radioactivity of receptors before and after exposure to unlabeled ACTH, the amount of unlabeled ACTH present could be determined.

Hormones from Lower Vertebrates and from Insects

Most of the studies supported by the Endocrinology program are concerned with mammalian hormones because of their obvious relationship to human medicine. Studies on non-mammalian forms however contribute to our overall knowledge, and often have surprising results, as with salmon calcitonin reported above. A conference on Comparative Endocrinology will be supported by NIAMD.

Studies of insect hormones approach some very basic problems. For example one grantee is studying the role of insect growth hormone on messenger RNA synthesis and the receptor mechanism whereby the hormone recognizes its target tissues. Such basic studies likely will yield clues which will be useful to human medicine.

05 - GASTROENTEROLOGY PROGRAM AREA

The Gastroenterology program encompasses research of organ systems associated with the gastrointestinal tract, including the salivary glands, esophagus, stomach, pancreas, liver, gall bladder, and small and large intestines. Research activity is concerned with the fundamental aspects of the physiological and biochemical processes of the normal and of disease. Efforts include studies of the cause, prevention, diagnosis, and treatment of specific diseases such as esophagitis, achalasia, peptic ulcer, pancreatitis, ulcerative colitis, Crohn's disease, malabsorption syndrome, gall stones, cirrhosis and hepatic coma; and basic and applied studies on relevant organ transplantation including techniques and storage.

Digestive diseases are the leading cause of hospitalization in our general population, produce more days of hospital stay than illnesses of any other body system, are the second major cause of days lost from work, and are the third leading cause of death. According to the U.S. Public Health Service, digestive diseases rank third among all medical and surgical conditions as a cause of economic loss, being exceeded only by cardiovascular disease and accidents.

Across the United States, 23% of all non-obstetric surgical operations are performed for digestive diseases. These operations include appendectomy, gastrectomy, cholecystectomy and colonic surgery. The total cost related to the 300,000 cholecystectomies performed annually approach a half billion dollars for this disorder alone. The cost of peptic ulcer alone during 1963 was estimated to be $1,000,000,000. If even 10% of the cost of these two disease entities could be saved (a modest estimate) by newer knowledge and widespread application of available knowledge, a saving of $150,000,000 annually could be realized.

A. PROGRESS IN GASTROENTEROLOGY RESEARCH

The following synopses are indicative of the diversity of research carried out both in the basic and clinical aspects of gastrointestinal disease with NIAMD support.

1. Gastrointestinal Hormones

From basic studies attempting to correlate the levels of serum gastrin with gastric acid secretion, NIAMD grantees (McGuigan and Trudeau) have established an important diagnostic test. This test readily distinguishes between two groups of patients, each of which has markedly increased rates of gastric acid secretion; those with the Zollinger-Ellison syndrome have elevated serum gastrins whereas those with peptic ulcer disease (usually duodenal) do not. They also observed that release of the hormone gastrin from the antral mucosa may be inhibited by acid. Thus, in pernicious anemia where acid secretion is impaired and the intragastric pH rises, serum gastrin levels are markedly elevated and in the range of patients having the Zollinger-Ellison syndrome. When the stomach is acidified by the administration of 0.1N hydrochloric acid, the serum gastrin levels can be reduced. Their basic observation of an inverse correlation between serum gastrin concentration and gastric acid secretion (when the gastrin is being secreted from the stomach and not from an extragastric site as in the Z-E syndrome), has provided an important diagnostic tool, and may provide a clearer understanding of the etiology of peptic ulcer disease.

Heartburn, caused by the reflux of acid stomach contents into the esophagus, is probably the most frequent complaint of gastrointestinal origin. More severe forms of this phenomenon occur in esophagitis and hiatal hernia. It has recently been found by an NIAMD grantee (L. Harris) that an important physiological structure, the lower esophageal sphincter (LES), which normally prevents stomach acid from entering the esophagus by contracting, is very sensitive to the hormone, gastrin. He has shown that individuals with incompetent LES who are susceptible to heartburn respond adequately to exogenous gastrin suggesting that the incompetency of the sphincter muscle is due to lack of endogenous gastrin rather than to an inability of the sphincter to produce increased pressure. The ingestion of antacids raises the pH in the stomach and releases the inhibition to gastrin secretion. The increased gastrin secreted then increases the tone of the lower esophageal sphincter. The relief of the heartburn may be mediated in part through gastrin release rather than wholly by the neutralization of stomach acids, as was formerly thought. Dr. M. Grossman, an NIAMD grantee, has postulated a one receptor-two active site theory which proposes that gastrin, cholecystokinin and secretin all act on the same receptor but have varying efficacies with relation to each other and to the effector organ. The rapid advancements made with NIAMD support in isolating and chemically identifying these three hormones provided the basis for predicting that gastrin and cholecystokinin, with chemically similar active fragments, would show an affinity for one active site whereas secretin has an affinity for the other. On the basis of experiments in which the individual efficacies of the three hormones have been determined on various target sites, the interactions of pairs of the hormones can be predicted and experimentally tested. Gastrin, acting at one receptor site, stimulates acid secretion in the stomach, whereas secretin, acting at the other active site is a non-competitive inhibitor of gastrin. Cholecystokinin, acting on the same receptor site as gastrin but with a lower efficacy, will compete for and competitively reduce the action of gastrin. This conceptualization and its experimental verification in man should go far toward allowing the development of useful blocking agents which fit the postulated receptor sites. Such agents are vitally needed in providing rational therapy for peptic ulcer, for example.

Measurements of circulating gastrin have been based on immunoreactivity. It has now been found (R. Yalow and S. Berson) that the major form of the circulating hormone is of much larger molecular weight than the biologically active heptadecapeptide synthesized by Gregory and Tracy, yet both give identical immunoreactivity. The importance of this "big-gastrin" and its variability in health and disease is currently being studied.

2. Gallstones

Human gallstones are composed predominantly of cholesterol. The other two major components (bile acids and lecithin) are necessary to solubilize the cholesterol in bile. In patients with cholesterol gallstones, Vlahcevic and Swell have shown that the bile salt pool is greatly diminished (to about one-half normal). They have also found that the excretion of lecithin into the bile is directly correlated with the bile salt pool size, whereas the excretion of cholesterol is only very slightly decreased by a reduced bile salt pool size. Consequently, these findings suggest that as a consequence of a reduced bile salt pool, gallstone-formers excrete less lecithin into the bile to solubilize the normal amount of cholesterol excreted.

Other NIAMD grantees (Thistle and Schoenfield) have attacked the problem of restoring the bile composition to normal in patients with cholelithiasis (gallstones) by administering an oral bile salt (chenodeoxycholic acid). In initial results, they found that the administration of chenodeoxycholic acid for one year was without toxicity, increased the chenodeoxycholic acid pool to normal, and decreased gallstone size by 70 percent.

NIAMD grantee W. Admirand has reported that the ability of various bile acids to solubilize cholesterol gallstones in vitro differs markedly, deoxycholate being the most effective, chenodeoxycholate next and cholate last. Conjugation of the bile salt decreases its solubilization of the stone; the taurine conjugates dissolve less cholesterol than do the glycine conjugates of the same bile acid.

3. Inflammatory Bowel Disease

Examination of the immunological and other aspects of Crohn's disease and ulcerative colitis has not yet disclosed whether these diseases are related or distinct processes; their etiologies are equally obscure. Both diseases show a familial and probably inherited tendency, and both have been found together within affected families. Polyarthritis, eczema, hay fever, and urticaria have been found more often in the relatives of colitic patients than in controls; the first three of these have been found more often in relatives of Crohn's disease patients. Several reports have shown that acute exacerbations of Crohn's disease have responded favorably to immunosuppressive agents. These associations suggest abnormalities of the immunological mechanism. Antibodies have been detected in the sera of colitic patients which react with an antigen derived from human colons. Similar antibody responses have been detected in Crohn's disease. Recently, epithelioid and giant-cell granulomas have been induced in the foot pads of normal and immunological deficient mice by injecting homogenates obtained from Crohn's small intestine and lymph node tissue. These lesions are similar to those induced by injections of homogenates of sarcoid tissue. Additionally, it has been demonstrated that the granulomas found in Crohn's disease are similar to those of sarcoidosis. These results may be regarded as indicative of the existence of a transmissible agent responsible for Crohn's disease, although, as yet, it has not been identified or isolated.

The incidence of Crohn's disease appears to be increasing in the United States, England, Scotland, Australia, and other countries. It is now a significant national health problem; in the U.S., 12,400 patients are hospitalized with this disease annually of which 3,600 are new cases. Because of its chronicity, the number of patients hospitalized each year is reported to be nearly four times the yearly number of new cases. Although the death rate from Crohn's disease is low, the morbidity is significant. In contrast to ulcerative colitis, in which total colectomy is curative, Crohn's disease of the small bowel or colon frequently recurs following surgical procedures. Because of its chronicity and the tendency for recurrence following surgery, there is a desperate need for effective medical therapy. Prednisone, a glucocorticoid, has been the most frequently used therapy, either alone or in conjunction with another drug, usually salicylazosulfapyridine (Azulfidine). Retrospective studies have demonstrated a favorable initial response to glucocorticoids, with many (50%) showing continued good results for six months or longer. Nevertheless, a significant number of patients (35%) still required surgery, and

only 10% of the patients were able to stop steroid therapy without recurrence. The incidence of steroid side effects is significant. Even less well defined is the therapeutic value of salicylazosulfapyridine. Despite absence of validated therapeutic data, this drug is widely used. Some retrospective studies show good responses in half of the patients receiving the drug alone or in combination with prednisone. Another drug that is increasingly being used in the therapy of Crohn's disease is azathioprine (Imuran), an immunosuppressive agent. Published reports indicate varying success rates and equivocal results. Objections to the use of azathioprine center around its potential hazards. A controlled study of its value is needed. The length of treatment, dosage, concurrent use of other drugs, toxic and long term effects of this drug, and criteria for disease activity indicating or justifying the use of azathioprine have not yet been established, despite which the usage of Imuran is increasing. A cooperative controlled study is urgently needed to establish the efficacy and safety of currently used drugs in the treatment of inflammatory bowel disease.

4. Liver Disease

The two major liver diseases, cirrhosis (on the increase in our adult population) and hepatitis (increasing at an alarming rate among our young population) are both crippling and fatal diseases of great social and economic importance. This is a rapidly moving field. There has been found an association between the presence of a viral-like particle referred to as the Australia antigen (Au) or the hepatitis associated antigen (HAA) and viral hepatitis (designated as Type B or MS-2 strain). It is now recognized that viral hepatitis is not only transmitted by parenteral administration of blood or blood products (gamma globulin) or by contaminated needles, but also by the respiratory or the gastrointestinal route. There is some evidence that fatal hepatic necrosis in cirrhosis follows only type B hepatitis, and that the antigen may be detected in the blood at some time during the course of the disease. Moreover, continued presence of the antigen in the serum may be related to chronicity of liver diseases. Klatskin of Yale University has extended his observations on the occurrence of HAA in various forms of chronic liver disease and has confirmed that the antigen can be detected in approximately 25% of patients with chronic active hepatitis and in 40% of patients with prolonged forms of viral hepatitis, but not in other types of chronic liver disease, including primary biliary cirrhosis. To confirm the latter Klatskin has analyzed sera from patients with primary biliary cirrhosis for the antigen by the immuno-electro-osmophoresis method and has not been able to confirm the work of Krohn that the antigen can be detected by this method. Studies will be continued using other immuno-electro-osmophoresis techniques to determine their sensitivity and reliability in detecting the antigen as compared with that of the standard gel diffusion method.

Because of the very high incidence of hepatitis after blood transfusion, several medical centers have initiated a screening procedure for the HAA in all donor blood, rejecting that found to be positive for the antigen. Results of such a screening procedure reported by Senior and Blumberg demonstrated a dramatic decrease in the incidence of post-transfusion hepatitis. Pre-screening studies showed that about 18% of recipients of whole blood or plasma developed anicteric hepatitis, and a few developed overt icteric hepatitis within four

to twelve weeks after transfusion. Early results of the blood screening has revealed a sharp decrease in the incidence of post-transfusion hepatitis, down to approximately 3% of the patients transfused. Screening of donor blood for the hepatitis antigen (HAA) may be practical and of greater value in high frequency-high risk groups such as the inner city, poor populations of metropolitan centers. Recognizing that it is often necessary to transfuse fresh blood before there is time to screen the blood for HAA using tests such as immunodiffusion or complement fixation which usually require overnight incubation, NIH grantees, Saravis and McDermott have developed a more rapid technique. Using a preformed microporous cellulose acetate membrane instead of agar, this electrophoretic method can detect the presence of HAA within two hours and is as sensitive as the Ouchterlony immunodiffusion technique. Recent improvements have further increased the sensitivity of this electrophoretic technique. It is important to investigate the clinical significance of the more than two-fold increase in the number of positive HAA sera detected by the new and more sensitive method.

Controversy continues regarding the role of blood supply in liver regeneration and the existence of a trophic factor in hepatic afferent blood. Interest in auxiliary liver transplantation in the treatment of end-stage cirrhosis, biliary atresia, and fulminant hepatic coma has focused special attention on the vascular requirements of liver transplants and the problem of "inter-liver competition" between the host liver and the graft. NIH grantees, Lee and Orloff have developed a technique of liver transplantation in rats to study the effect of blood supply on regeneration of heterotopically transplanted 30% hepatic isografts, free from the complicating influences of immunologic rejection and immunosuppressive therapy. It was found that grafts nourished by both portal vein and hepatic artery or by portal vein alone regenerated signicantly. Liver transplants, however, that lacked a portal venous blood supply atrophied under all circumstances even when the host liver was also deprived of its portal venous flow. It is concluded that portal venous blood plays a specific role in liver regeneration and in maintaining nutrition of auxiliary liver transplants. In contrast to this conclusion, McDermott et al. have found that satisfactory liver function and regeneration can occur in the absence of afferent portal blood supply. In dogs that were subjected to a standardized left hemi-hepatectomy, the hemodynamic inflow to the remaining half of the liver was varied in different groups of subjects to define the physiological requirements for regeneration and function of the host liver. It was found that there was no statistical difference in the degree of regeneration between groups of animals having portal vein and hepatic artery, portal vein alone, or renal vein and hepatic artery. There was a difference when there was only hepatic artery inflow. It was concluded that adequate total blood flow was the important factor for regeneration, not a specific portal substance. If a specific hepatotrophic factor were present in the portal blood, it was either completely or partially removed or utilized by non-hepatic organs of the body and/or required a minimum blood flow to the liver which was greater than that supplied by the hepatic artery alone.

Additional evidence that a portal blood factor exists as a humoral agent effecting liver regeneration was offered in a recent report by NIH grantee Fisher. The data obtained from his studies demonstrated, by use of extra-corporeal cross circulation, that the humoral factor responsible for liver

regeneration does not arise from the liver remnant of a partially hepatectomized animal. Intact livers of normal rats showed an increase in the specific activity of DNA in hepatic parenchymal cells in proportion to the amount of liver removed in the parabiotic partner, with the greatest response (fivefold increase) occurring after total hepatectomy. Evidence from portacaval-shunted, partially hepatectomized rats connected to normal members indicates that the factor is in portal blood and that the onset of regeneration is the result of a quantitative imbalance between the available portal blood factor and the number of liver cells present. Further investigations are planned to determine the nature, site of origin, mechanism of action, and regulation of concentration of the portal blood factor.

B. PROGRESS IN GASTROENTEROLOGY TRAINING

During the past year, 39 training grants in gastroenterology have been supported in twenty states for a total of 2.25 million dollars. Ninety trainees have received full-time training in clinical research, teaching and special clinical procedures during the past year.

In addition to reviewing 16 training grants, three supplements, and 23 fellowships in the two specialties, the training committee for Gastroenterology and Nutrition held a series of discussions, reviewing its functions. At the September meeting, the recent history of approved training programs in gastroenterology was considered with respect to their success rates. At the January meeting, a reevaluation of the purposes of training programs, the number and kinds of training programs needed, and factors other than training programs which are required for development and retention of academicians in gastroenterology and nutrition were discussed. The major goal of the training programs continues to be the training of physicians for full-time academic positions in these two sub-specialties. The committee was of the opinion that, at present, there exists a sizeable gap between the end product of the training program and a faculty member who can function effectively in the three areas required; teaching, patient care, and research. Research ability, which is essential for stimulating the accumulation and dissemination of knowledge, is the most difficult goal to reach for the present training program graduates. Without adequate continuing support, many promising trainees are discouraged from pursuing careers in academic medicine at the termination of their traineeships.

There was a unanimity of opinion that even under the most favorable of training environments and with highly qualified trainees (those who have finished their residency, training in the sub-specialty, and a limited amount of research experience) the usual one or two years on the training grant is not sufficient for them to become established as independent investigators. Their opportunities are exceedingly limited; they are not yet ready to compete for NIH research grant support; support from currently active research programs is limited because of reduction in available funds from all sources; and they are too many years away from being ready to obtain support as Research Career Development Awardees. Simply extending the years of support to encompass a training experience of five years rather than two years is not realistic from either the prestige or monetary standpoint for these individuals with five or more years post-doctoral experience. While admittedly still

requiring several years with some guidance to develop into mature faculty, they also need some sense of independence, position security, and advancement. Salary support plus some research support was considered to be minimal requirements. The committee was of the opinion that the number of such positions should be quite limited, competitively selected, and that salary should be commensurate with junior faculty positions. Salaries and recognition should be commensurate with these high standards.

The discussion of this committee resulted in the drafting, by NIAMD staff, of two new support instruments to meet real needs in the fields of gastroenterology and nutrition. Their presentation to NAAMD Council was received enthusiastically. These new support instruments have been approved by the Office of the Director, NIH, for implementation. These two awards would be limited to the areas of gastroenterology and nutrition. They are described in the following paragraphs.

The three-year award, tentatively called the Clinical Investigator Award, would be to provide the opportunity for promising young physicians with demonstrated aptitude in research to develop fully into independent investigators under the supervision of appropriate sponsors. This instrument would provide an attractive position in shortage categorical areas which would allow the maturation of young health professionals into academic careers with research as a major component. The proposal emphasizes the need to retain excellent people in the research-academic career track by a) early recognition of potential (by the giving of this award), b) making possible longer term, better planned, and more diversified research experience, and c) salaries designed to minimize the present disadvantage in relationship to the other high income alternatives of these health professionals. The award would support the applicant and provide a modest supply allowance for his research for three years. It would be non-renewable and non-transferable.

A second award, especially needed in the area of nutrition which suffers an identity crisis, is tentatively called the Academic Career Development Award. The purpose of this award would be to provide the opportunity for accomplished young physicians in seriously short categorical areas to bring their expertise, interest and development into a potentiating role with plans of individual institutions to remedy deficits in those specific categorical areas. A five-year period of support at a junior faculty level would be provided in an institution with a demonstrated great need within a specified categorical field for the following purposes: a) initiation or augmentation of that categorical interest, b) development and support of teaching efforts, and c) the joint support of the applicant's career development and the substantive field within that institution. Emphasis would be on the ability of this individual to develop the categorical effort within the institution and to ally those categorical activities with other sub-specialties, and on the further development of the applicant as an educator and clinical scholar. The award would support the applicant's salary, be non-renewable and non-transferable and be limited to one such award in each field per institution.

The GIN committee also proposed a new type of training program, an interdepartmental gastroenterological training program at the senior postgraduate level. The program would be intended to provide a small and highly select

group of individuals with two to four years of specialized training in gastroenterology, including gastroenterological surgery, radiology, and pediatric gastroenterology in direct preparation for a full-time academic position. For acceptance into the program, a candidate would have completed his house officer training; had two years as a trainee or fellow in gastroenterology (in medicine or pediatrics) or board certification in surgery or radiology; demonstrated talent and motivation for an academic career; demonstrated effectiveness in teaching at an undergraduate and postgraduate level; and shown originality and proficiency in clinical or basic investigation. The number of trainees would be strictly limited to no more than one per participating department and the training period would be from two to three years at stipends conforming to an appropriate position or rank in the medical school. NIAMD staff has not yet pursued the possibility of implementing this kind of proposal.

In essence the GIN committee tried to point the way toward much needed innovations in training to fill gap areas, thereby enhancing the effectiveness of the existing training programs, as well as providing an additional dimension for supplying the academic community with well-trained gastroenterologists and nutritionists.

Introduction

Projects involving erythropoiesis and the metabolic intracacies of the immune response continue to form the bulk of the Hematology program. These two areas account for 75% of the research projects and 90% of the research efforts of investigators supported by RCDAs and RCAs. In ongoing programs the predominant area of effort has been in erythropoiesis, whereas starting grants dealing with the immune response equal in number those in the area of erythropoiesis. One factor contributing to this shift of emphasis is the stimulus of clinical organ transplantation activity and the advances in the attendant areas of histocompatibility testing and the graft-host mechanism that have occurred in the past decade.

Over the past year, an increased effort has been directed towards learning more about sickle cell disease. Many investigators studying abnormal hemoglobins are now utilizing hemoglobin S as a model, and some general studies in the areas of hemolytic anemia and granulocyte kinetics are concentrating on those aspects of sickle cell disease. The opportunity for an experienced and capable bio-medical investigator to respond to the increased interest in sickle cell disease reveals the advantage of the research grant support mechanism.

Erythropoiesis

Iron Metabolism - Although iron metabolism has been one of the most extensively studied aspects of hematology, many of the biochemical steps essential to normal iron utilization remain unknown or incompletely understood. The pathways that iron follows within cells and how iron enters and leaves cells are obscure.

Fruitful studies underway involve the use of experimental models in which iron metabolism is disturbed, including copper deficiency, vitamin B6 deficiency and hemorrahagic anemia in swine. A defect in heme biosynthesis has been observed in copper deficiency which explains the hypochromic, microcytic cells that are produced, the accumulation in the cytoplasm of iron not utilized for heme synthesis, and the reduction of incorporation of transferrin ^{59}Fe into heme by reticulocytes. It has been shown that the copper protein in plasma, ceruloplasmin, has ferroxidase activity and is thus able to catalyze the oxidation of ferrous to ferric iron which then binds with apotransferrin to form transferrin whereby iron is transported to the hemoglobin synthesizing cell. This finding has important implications in the nutrition of iron-deficient individuals.

Steroid Hormone Metabolites - Several 5 beta-H steroid metabolites of the hormones testosterone and progesterone have been shown to influence erythropoiesis by what appears to be an enzymatic pathway. Delta-aminolevulinic acid synthetase formation is apparently enhanced by these metabolites and the rate of heme production is thus increased. Plasma erythropoietin levels were not increased by these metabolites which suggests that erythropoietin was not responsible for the observed erythropoietic activity.

Folate Binding Factor - One of the vitamin coenzymes necessary for DNA synthesis is folic acid. The role of this vitamin in preventing megaloblastic anemia has been known for some time. Recent investigations have shown a macromolecular factor in the lysates of the peripheral cells of some patients with chronic myelogenous leukemia and acute lymphocytic leukemia and several pregnant women that binds folic acid. The function of this factor is as yet unknown but based on the knowledge of folic acid's essential role in DNA synthesis a physiologic or pathologic relationship is suggested. Further, current studies show that methotrexate is also bound by this factor which binding may, like that of folate, inhibit reaction with folate reductase and thus be the mechanism by which leukemic cells become resistant to methotrexate. Discoveries of this nature illustrate that investigations into basic mechanisms often may provide important information useful in other bio-medical areas.

Hemoglobin Structure - Defining the molecular structure and amino acid substitutions in various hemoglobins and how these structural variants influence the oxygen carrying ability of these proteins are efforts that are actively supported by NIAMD. The basic understanding of structure-function thus gained often provides insights into areas other than the obvious one of oxygen transport. A recent study that illustrates this point is one in which the proportions of hgb A_{1c} were measured in diabetic and normal individuals and found to be increased in the diabetics. Characterization of hgb A_{1c} showed it to be a glycohemoglobin and its increased level in diabetics could be correlated with the increased amounts of unusual glycoprotein found in the basement-membrane material of their kidneys.

Enzymes

Pyruvate Kinase Deficiency - A recent study has explained the mechanism by which pyruvate kinase (PK) deficient red cells are selectively removed from the circulation. PK deficient reticulocytes were shown to be sequestered and promptly destroyed by the reticulo-endothelial system (RES) whereas older PK deficient red blood cells survived longer. Low rates of glycolysis and therefore greater reliance on oxidative phosphorylation for ATP production were shown to be characteristic of PK deficient reticulocytes. Reduction in the level of intracellular ATP allows the cell to lose potassium and water giving rise to a shrunken, spiculated, viscous cell with rheologic properties favoring sequestration by the RES.

2,3 Diphosphoglycerate and Anemia - A new role for 2,3-DPG in anemic individuals was shown in a study relating levels of this enzyme to oxygen-hemoglobin dissociation rates (p50). Normal and anemic individuals were studied and it was found that as hemoglobin diminished in concentration, 2,3-DPG levels rose, paralleling a rise in the p50. The results showed that 2,3-DPG induced changes in hemoglobin affinity for oxygen may compensate for up to one-half the oxygen deficit in anemia.

Immune Response

Transplantation Immunity - Studies on bone marrow allografts may provide information significant to organ transplantation in man, particularly for

clinical hemopoietic cell transplantation. This type of replacement could become a therapeutic procedure in diseases like sickle cell anemia, aplastic anemia, acquired agranulocytosis, inherited and acquired defects of the immune system and leukemia.

In mice, studies have indicated that the host parameters of the allograft rejection are different for hemopoietic and for skin grafts. This new finding could influence current thinking in the selection of organ transplant donors which has depended heavily on histocompatibility testing using peripheral blood cells as test cells, which in turn assumes they have antigens in common with tissue cells.

In a study of the antibodies in immunosuppressive antisera made against antigens on various blood cells, it was found that although such cells as erythrocytes, granulocytes, lymphocytes and thymocytes may be very closely related, they need not carry common antigens. This finding could lead to the development of highly specific immunosuppressive antisera that could be used against mature lymphocytes, preventing graft rejection, without damaging other cell lines.

Another example of investigators developing basic knowledge and techniques in one area of work which have then been utilized to gain insights into another biomedical area concerns studies of lymphocytotoxins. In investigating the serum of patients with autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis and with the virus diseases rubella, measles, and infectious mononucleosis, it was found that lymphcytotoxins had developed "spontaneously" as opposed to a response to allogeneic immunization. The biological significance of this finding is still unknown but it may be the orized that if lymphocytotoxins are active in vivo in autoimmune states, they could be lysing cells which then release nuclear and cytoplasmic elements which may evoke the formation of antinuclear antibodies.

Leukocytes

Neutropoietin - With the use of an in vitro soft agar technique for growing granulocytic clones, it has been shown that white cells do produce a factor that stimulates growth of these cultures. This finding is providing the base for the search for neutropoietin. The clear demonstration of a factor controlling neutrophil production would be of great interest as to its possible role in such diseases as leukemia.

Lymphocyte Transformation - The demonstration of in vitro stimulation of lymphocyte blastogenesis by soluble antigens from malignant melanomas in a number of patients have established that these patients have a common antigen(s) in their tumors. An extension of these studies has shown that a patient's lymphocytes can be transformed by the stimulus of antigen(s) from that patient's own tumor.

Training

Since 1967 when there was a rather sharp decline of five in the number of hematology training grants supported, thirty-eight programs have been funded.

Four new programs were started, one in 1968, three in 1969 and offset the loss of four previously supported programs. Two of the new starts were programs designed to train pediatric hematologists and represented a modification of the program's objective to include pediatric hematology as part of the adult hematology service rather than a separate sub-speciality.

Close to 80% utilization of available training slots for postdoctoral trainees was observed for the programs as a whole. The observation that those eight programs which at the last review had received the best priority scores (150 or better) accounted for a disproportionately large fraction (39%) of all the NIAMD supported postdoctoral training in hematology shows the attractiveness of these programs to potential trainees. These same eight programs, with scores of 150 or better, also had the best records of graduates attaining academic rank.

Career Development Program

The number of active awards in this area declined in the past year from 21 to 18. These individuals, supported in 13 different institutional environments in the United States represent NIAMD's efforts to encourage the development of biomedical investigators in the area of hematology. In addition to their research projects in such areas as megakaryocyte and platelet kinetics, immunochemistry of hemoglobin variants, the absorption and distribution of iron, and the regulation of hemoglobin synthesis, most are involved in teaching medical students and providing patient care in an academic setting.

The Metabolism Program includes approximately one-fourth of all research grants and the largest fellowship program of the Institute. It includes a large number of "one-of-a-kind" projects which defy simple classification. Larger programs concern transport of sugars, amino acids, and electrolytes; the general aspects of cyclic AMP metabolism including adenyl cyclase, protein kinase, and the regulation of phosphorylase and glycogen synthetase; general mechanisms of protein synthesis including packaging and release; ketogenesis; gluconeogenesis; fatty acid synthesis; mitochondrial metabolism; oxidative phosphorylation; oxygen metabolism including the hydroxylation and oxygenation reactions; and abnormalities such as galactosemia, gout, the glycogenoses, and particularly, Cystic Fibrosis of the Pancreas.

Significance of Fiscal Restraints

The plateauing of research grants funds in the face of increased costs of carrying out research has resulted in loss of grants to the point that many crucial projects have been endangered or lost to the program. The review groups and Council have recognized the importance of giving the newly-established investigator, usually the superb product of a training program, support to demonstrate his productivity but this is possible only with termination of support of more established investigators who have not been overly productive. How serious the situation can be is illustrated by two cases in which the initial application did not result in a continuation award but which won enthusiastic approval on second review. Fortunately, substantial breakthroughs in the problems were made before the projects had to be terminated. One project promises to clarify insulin's mechanism of action at the cell membrane in controlling the availability of powerful metabolic regulators to subcellular organelles and the cytoplasm. The second case concerns the mechanism of oxidative phosphorylation which has continued to be elusive despite the isolation of coupling proteins, reasonably detailed mapping of the various respiratory enzymes in the mitochondria and identification of the electron transfers coupled to phosphorylation. Not only was the generation of high energy phosphate coupled to the oxidation of glutathione by cytochrome C demonstrated but also uncoupling by a variety of agents at the same concentration of uncoupler effective in the natural systems. These findings focus attention on a three-membered ring of substituted sulfur atoms which can undergo oxidation to a free radical high energy intermediate which is subject to attack by inorganic phosphate or by oxygen. The simple system can be studied only anaerobically and with strict avoidance of ionic copper or iron which can short circuit the reaction.

Part of the importance of these observations is in understanding ion transport which can be coupled to high energy phosphate and the generation of peroxide accompanying the fusion of lysosome and phagosome. Both mitochondria and the ATP dependent ion pumps can run backwards, exchanging ion energy for phosphate bond energy. These reactions, however, occur in membranes and much of the function of the membrane remains to

be understood in terms of protecting the high energy intermediates including free radicals from attack by water and oxygen.

Affinity Chromatography

Outstanding among the new projects was the continuation extramurally of studies of affinity chromatography initiated intramurally. Affinity chromatography takes advantage of the exquisite specificity of binding between enzymes and their substrates or inhibitors, antigens and their antibodies, hormones and their receptors and carrier proteins, and polynucleotides and their complementary strands. With attachment of one member of the binding pair to a stationary support such as sepharose the other member can be separated from complex biological materials simply and specifically. What is most surprising, perhaps explaining the slow recognition of the potential of the method, is that relatively simple compounds such as cyclic AMP and steroid hormones can be derivatised for bonding to the supporting medium without serious loss of affinity for its receptor.

Understanding Disease

The heart of the Metabolism program consists of the many long-term projects which continue to turn out the basic data necessary to understanding metabolism. In the 518 awards with fiscal 1970 funds, two hundred twenty went to projects in their fifth or greater (with individual awards in years 24 and 19) year at the same institution. From such projects has come the basis but not the culmination for such headline announcements as the immunoassay for a colon cancer antigen which is also detectable in foetal life. One long-term metabolism project has demonstrated a decrease in sialic acid groups in the surface antigens of virus transformed cells while another has demonstrated the loss of the sialyl transferase from the membrane under similar circumstances.

Membrane Research

It is in the field of membrane metabolism that the most interesting results are arising and the future should hold many more exciting developments. The question is both what these developments will be and when will they occur. The field is still in its descriptive phase with much expenditure of effort to obtain sufficiently pure membranes to justify morphologic and chemical characterization. There is considerable question, however, if the final product bears much resemblance to the native membrane, particularly as regards function. Some of the most interesting membranes such as endoplasmic reticulum, Golgi apparatus, and secretory granules are undergoing constant synthesis, degradation and remodeling so it is not surprising that most membranes are now characterized by a distribution of enzymatic activities rather than chemical composition or function.

Cystic Fibrosis

Much of the pathogenesis of many diseases including diabetes, Cystic Fibrosis, and hyperthyroidism is thought to occur at the membrane level

and it is doubtful if this metabolism can be adequately understood until better methods are available to study membrane function. Diabetes and Cystic Fibrosis have in common comparable microvascular pathology, delayed initial insulin release in response to hypoglycemia, and changes (in CF at least in the tissue culture fibroblast) in the activity of enzymes controlling hydroxylation and glycosylation of collagen. It is also noteworthy that the Beta cells of the pancreas can be destroyed by the prooxidant alloxan in a poorly understood series of reactions in which trace metals have a strong influence while Cystic Fibrosis is known for its high requirement for tocopherol and the tendency for lipofuscin deposition. Cystic Fibrosis is characterized by the cilia disturbance effected by an IgG serum factor while a comparable factor (also an IgG) is the long acting thyroid stimulator of some hyperthyroid conditions. It seems apparent that there are factors in all three conditions which influence membrane metabolism and a breakthrough at this level in one disease will contribute to understanding the others.

A host of questions regarding the IgG factor in Cystic Fibrosis and its relation to the pathogenesis of the disease and to the abnormal gene remain unanswered because the necessary studies cannot be carried out on man and an animal model of the disease is yet to be developed. Rapid progress can now be expected with demonstration of the reliability of the oyster gill assay for the IgG factor and the demonstration that the factor is produced by the fibroblast in tissue culture. Thus it should soon be available not only in quantity but tagged with radioactivity for animal studies. The present degree of purification may be sufficient for studies of its pharmacologic effects. Definitive metabolic studies must await further purification. This appears promising with precipitation of some of the non-factor IgG with antibodies to normal human IgG. Fluorescent antibody is being used in search for the combining site on the oyster gill and this study could lead to an affinity chromatography separation of the factor.

The development of an animal model for Cystic Fibrosis remains an important objective which may now be feasible with the oyster gill assay. The assay is too time-consuming for study of other than the most probable carriers of the abnormal gene. A simple chemical marker is needed for any screening of large numbers of animals and may be developed in relation to the abnormal tocopherol requirement of the cells.

Endocrine studies continue to turn up interesting findings suggestive of a markedly blunted response of end organs to a variety of hormones. Sweat electrolyte levels remain elevated despite elevated levels of aldosterone and aldosterone secretion does not suppress with high salt intake. Intramural investigators have demonstrated apparent failure to suppress parathormone with calcium infusion, perhaps explaining the low bone density. Massive doses of secretin are necessary to effect a

normal pancreatic electrolyte response in thirty minutes. There is also a response of both the sweat electrolytes and of growth with massive anabolic steroid therapy but perhaps an accelerated deterioration in pulmonary function. In two separate sets of adrenals obtained at autopsy, a marked decrease in the conversion of progesterone to cortisone and increase in the mineralocorticoid pathway has been demonstrated. As the serum factor and its neutralizing antibody become available it should be possible to sort out the effects of the factor from possible inherent membrane abnormalities.

With this background of information it is easy to recognize the difficulty in determining the value of various supporting measures in CF. Tachyphylaxis to a variety of hormones is apparent and may represent severe limitation in the availability of a factor or factors involved in the mechanism of action of the hormone. Thus restoration toward normal of one endocrine end organ effect may further limit other more important effects. Methionine, selenium, and tocopherol appear to work synergistically both in controlling lipid peroxidation and in avoidance of pathological degeneration of the pancreas and liver. Methionine is an essential precursor of spermine and spermidine (with fundamental roles in organization of the polysome complex); the methyl groups of histones, transfer RNA, DNA, and choline; and of homocysteic acid with its apparent influence on cartilage sulfation. It is noteworthy that isoproterenol, the massive prolonged administration of which to the rat induces a CF like syndrome including appearance of the serum factor, not only stimulates the enlargement of salivary glands by proliferation of acinar cells but places demands on the methyl pool through the O-methyl transferase reaction.

It is known that lipid peroxidation as reflected by lipofuscin deposition can be limited by massive administration of tocopherol. The Cleveland group using such supplementation along with other intensive supporting measures has a remarkably good mortality and morbidity record. These results have required years of careful study. What is most needed is enough insight into the pathogenesis of the disease to recognize those metabolic patterns which are protective and those which are deleterious. The promised greater availability of the serum factor from tissue culture should facilitate such developments.

Further advance in CF research will depend on observing the metabolic influence of the serum factor on characteristic mammalian membranes rather than such a foreign target as the oyster gill. Presumably calcium transport could be involved since its metabolism appears to be abnormal in more than one respect in Cystic Fibrosis.

Free Radical Pathology

A second area of continued interest will be the regulation of free radical reactions since there is evidence for lipid peroxidation in the deposition of lipofuscin and increased requirement for tocopherol. Essentially every electron on its transport to oxygen appears at some time in the form of a free radical, primarily in the flavin enzymes and

secondarily in the non-heme iron labile sulfur carriers. An important feature of the membrane of the electron transport chain is to block attack of water on the free radicals and any extraneous activation of oxygen. The membrane contains not only lipid but copper and iron proteins which can undergo one electron changes in oxidation state. A free radical is characterized by an unpaired electron and is neutralized on pairing with an electron from a metal such as copper or iron.

The membrane functions to divert electrons (equivalent to free radicals) from the transport chain for various synthetic and degradative reactions. The various hydroxylation reactions of phenylalanine and its metabolites and of the sterols are typical of the synthetic reactions while the generation of peroxide in the fusion of the lysosome and phagosome are characteristic of degradative reactions. The dithio exchange reactions of a remodeling membrane may generate free radicals (comparable to the glutathione-cytochrome C reaction discussed earlier). The function of the trace lipids (vitamins A and E) remains a mystery but they may serve to keep free radicals under control during the introduction of the flavins and metalloproteins into membranes. Thyroxine has anti-oxidant activity and one of its effects at physiological concentration is to increase the flavin content of various cell membranes. Much additional information is also needed on the effect of free radicals on cleavage reactions. The dipole of a functional group determines the attack of water. A free radical, representing a charge difference of one electron, should markedly alter the kinetics of a variety of cleavage reactions and could explain the catabolism of a number of compounds such as heparin for which mammalian enzymes are unknown.

At no time in history has there been greater awareness, on the part of citizens-at-large, of the vital relationship of nutrition to human health, the extent of undernutrition and malnutrition in all countries of the world, and the problem of imbalances between world food supply and population growth. To meet these challenges there is urgent need for expansion of nutrition research, both basic and clincal, and for increased training of physicians and paramedical personnel in the best possible use of nutritional knowledge toward the prevention and treatment of disease. Of the various institutes of the National Institutes of Health, the National Institute of Arthritis and Metabolic Diseases has, by virtue of its mission, the greatest responsibility in fostering research and training in nutrition most needed to meet these national and international problems. Regrettably, during fiscal 1971 the nutrition program has been obliged to maintain its status quo.

On the other hand, encouraging progress has been made in planning for future operations of the program. First, there are plans for a closer affiliation of the programs in Gastroenterology and Nutrition under a combined title of Digestive Diseases and Nutrition. Second, approval has been obtained for implementing two new instruments, a Clinical Investigator Award in DD-N and an Academic Career Development Award in DD-N, specifically related to training investigators and teachers, and designed to strengthen and supplement the existing training programs in these areas.

Research Grant Support

Comparing fiscal 1971 to fiscal 1970, there has been a small increase in applications directed to Area 8 (130 vs. 125), a small decrease in approved grants (73 to 79), and a significant decrease in those funded (28 vs. 36). Of the latter, 11 represent new grants and 17 renewal requests, as compared to 15 and 21, respectively, funded in fiscal 1970. In addition to 4 currently active program projects, one was phased out, one was renewed, and one new project was approved and funded.

Trends and Developments in Supported Research

Of the broad spectrum of research supported through the Nutrition program, increased attention has been focused upon four major areas.

1. Overweight and Obesity. The cause, prevention and treatment of overweight and obesity is an important health problem in our adult population. Studies supported are concerned with morphogenesis of adipose tissue cells, aberrations of lipid metabolism, value of medium chain triglycerides, role of unsaturated fatty acids, body composition and multiple factors regulating appetite. Steady progress has been made through operation of 26 grants having relevance to these problems.

2. Vitamins. The mechanisms of action and metabolic functions of the fat-soluble vitamins A, D, E and K are being studied. Notable advances have been made toward identification of the specific retinol-binding protein so important in plasma transport of vitamin A. A most interesting breakthrough

occurred during fiscal 1971 with the discovery that it is in the kidney, not in many other tissues previously suspected, that 25-hydroxycholecalciferol, the major biologically active form of vitamin D, is converted to a metabolite several times more active. The absence, or decrease, of kidney enzymes necessary for this conversion, may explain the resistance to the action of vitamin D observed in osteodystrophy of chronic renal failure and in familial rickets. Some 26 research projects are being supported, as compared to 16 related to water-soluble vitamins. The latter are rather equally distributed between vitamins B_1, B_6, B12, and biotin and riboflavin.

3. Proteins and Amino Acids. Protein requirements of man, the metabolic role of amino acids, and effects of protein-calorie deprivation are receiving major consideration. There has been good progress, but no outstanding achievements.

4. Role of Trace Elements. There has been an increase in requests for support of research on the biological role of trace elements (zinc, selenium, copper, magnesium and chromium). Increased consumption of more highly purified cereals and other dietary components is relevant to this interest, especially since there are recognized more than 200 enzymes and coenzymes containing one or another trace element as an essential constituent. Certain elements function also as activators of enzymes. Important studies are in progress designed to determine the incidence and clinical importance of chromium deficiency in juvenile diabetes, gestational diabetes, and certain disturbances of carbohydrate metabolism, in the hope that such conditions may be responsive to the "glucose tolerance" factor, a chromium-containing organic complex.

U.S.- Japan Cooperative Medical Science Program

In addition to the research grants previously discussed, this special program has provided support for 16 research projects administered by NIAMD, as well as 4 others assigned to NICHD. The NIAMD projects relate to: 1) nature and treatment of marasmus and kwashiorkor, 2) interrelations between nutrition and infection, 3) anemias in pregnant women and young children, 4) protein requirements of Orientals, 5) low cost vegetable supplements for infants and young children, 6) uptake of iron from cereal diets, 7) improved protein and lysine content of cereal grains, and 8) toxicity effects of food mycotoxins. Results of the first five years of grant and contract sponsored research recently have been published in a complete, and also an abbreviated, report.

Over the past three years this program director has, through functioning as secretary of the Malnutrition Panel, had general "watch-dog" responsibilities for the activation and operation of both NICHD and NIAMD research grants supported by this program, while the funding of grants has been the responsibility of NIAID. It is gratifying to note that a most harmonious and effective relationship has been maintained by the staff members of each of the Institutes involved. It is understood that funds previously allocated by NIAID to support research grants of this program will be included within the budget of NIAMD, beginning with fiscal 1972.

Training Grants and Fellowships

The 11 training grants in Nutrition, one less than last year, have supported 17 predoctoral and 16 postdoctoral trainees, totaling 13 less than last year. One new grant, to be activated July 1, 1971, will replace one terminating this year. There are prospects of two new applications for training grants for consideration by November 1971 Council. There is need for much missionary work to be done in creating greater interest in nutrition research and training, and in fostering a stronger image of nutrition, in our medical schools. The two new training instruments implemented by the Digestive Disease and Nutrition programs represent a definite step in this direction. There is an urgent need to encourage and support young men with potential for developing training programs, for most of the present training program directors are gradually reaching "senior citizen" status.

Eight postdoctoral fellows, 2 less than in fiscal 1970, have received support. The number of RCDA awards (6) and RCA awards (3) have remained the same. Both the number and quality of new applicants have been disappointing. It is felt that this reflects not so much the lack of promising young candidates as it does lack of awareness in the "field" generally of support available for this type of training.

Outside Activities

In addition to assigned duties and responsibilities, the Program Director has participated in the following:

1. As NIAMD representative at monthly meetings of the Interagency Committee on Nutrition Education, and member of its subcommittee on Evaluation of Agency Recommendations on the Purposes and Functions of ICNE.

2) As a member (19th year) of the Scientific Advisory Committee of the Muscular Dystrophy Associations of America in its biennial review of research grants and fellowships, and as Member of the Board of Directors of this health agency.

3) As a member of the Washington, D. C. Area Trace Element Club.

Electrical Effects on Bone Growth

The effect of applied current on bone growth is an area of controversy among investigators. Noteworthy results have been reported recently in this field. Using tiny charges of electricity in tissue culture systems, two types of human cells (stem and lymphocytes) have been induced to produce masses of blastocytes. Morphological and cytochemical differentiation of human lymphocytes has also been produced. These cells become more sensitive to electrical current if pretreated with prolactin. Recent investigations have indicated an increase in the rate of growth and possible fracture healing of mammalian bone under controlled electrical stimulation.

Implant Research

Research in implants continues to be an area of great interest and activity. Investigation of ceramics, various metals, metal alloys, carbons, and plastics as potential implants has produced data of importance. Although tissue has been shown to grow into and infiltrate pores of ceramic implants, this union has not been long-lived due in part to the relative brittleness of the ceramics and to the slow dissolution of the material and resultant pH changes *in vivo*. Corrosion and wear studies of vitallium, 316L stainless steel, unalloyed titanium, and zirconium alloy indicated no corrosion had occurred twelve months after implantation. Materials were implanted in the solid and powder forms. Preliminary results of implants of fiber reinforced epoxy resins (graphite and quartz fibers) have shown tissue reaction comparable to commonly used metal implants. Wear studies are being conducted *in vitro* using combinations of metal on metals, metal on plastics, plastic on plastic and reinforced epoxy resins with the above. An ultrahigh molecular weight polyethylene RCH-1000 has shown the highest resistance to wear of the plastics tested, and vitallium appears to produce the least wear when coupled with plastics. Testing of material will be continued and extended.

Short-length metal fibers or wires have been molded to desired shape, sintered, and implanted in animals. These implants were rapidly invaded by connective tissue, blood vessels and bone. This invasion occurred within three weeks and was still viable at six months. The bonding between tissue and implant was comparable to that between methacrylate and bone. The ease of preparation of molded metal fiber implants, the apparent lack of tissue reaction and the early invasion of these implants by tissue cells makes these materials appear extremely promising. Long-term studies of implants will, however, be necessary to properly evaluate the usefulness of this material in the human.

Other Research

Specific discoveries recently reported that may be of major importance are:

1. A new autoradiographic method for quantitating total uptake and regional distribution of radiocalcium in microscopic sections of bone.

2. Induction of osteoporosis in hypophysectomized mice and rats by the repeated injection of parathyroid extract. The extract apparently stimulates the osteoclastic potential of the osteocytes directly rather than indirectly through growth hormone or thyroid activity.

3. Demonstration that adipose tissue is the principal mammalian storage site for biologically active vitamin D.

4. Procedures permitting measurements, for the first time, of the total bone resorption rate and the linear bone resorption rate.

5. Preliminary data suggesting that increasing concentrations of calcium in the diet may decrease the net absorption of vitamin D.

Research is continuing on connective tissue, cartilage, osteoporosis, and similar areas of interest to orthopedics.

10 - PHYSICAL BIOLOGY AND RELATED AREAS PROGRAM

This program area, formerly designated as "Related Areas," was given the present, more descriptive name, to identify more accurately the character of the research projects supported within it. During the past year substantial results have been reported in these studies, which in the main correlate biological and physical structure of proteins, lipids, carbohydrates, enzymes, etc., with their function in living forms.

New methods are being developed for protein synthesis and its application to structure-function relations which should provide a number of direct potential health uses. For example, the blood and other fluids contain a number of proteins which have direct effects on a myriad of biological functions. Proper application of synthetic methods to the preparation of a desired protein which the body does not, or for some reason cannot synthesize would make the protein available for that individual. Since the protein would be identical to that ordinarily present in the individual, it would provide a return to normal function. A possible use in the future would be for specifically modified synthetic proteins that could provide very specific reactions in the body. As in many cases the various proteins mentioned above are not available from natural sources or are only available in minute quantities, a vastly improved synthetic methodology over that currently available is highly desirable. Further, a more precise understanding of structure-function relations in proteins is needed to appreciate fully the biological roles of proteins. Many desired compounds for these studies can only be obtained by synthesis of the protein.

One of our investigators has focused his attention on the discovery, development and refinement of synthetic methods for the production of modified steroids that may be of therapeutic use. The fine tuning of human biochemical processes through the administration of natural and synthetic steroids continues to be a major area of therapy which has led to many diverse modifications of the steroid structure in order to gain selectivity in physiological action. Indeed, dramatic effects have been achieved by the use of rather grossly modified steroidal molecules, i.e., the pill.

Experimental studies are being carried out to characterize the relative tendency of an amino acid to adopt either a helical or a coil formation. These data may provide a sound basis for analyzing the stability of various conformations in proteins. A quantitative basis may be derived for identifying the regions in the amino acid sequence of a protein where the nucleation of the folding of the protein chain occurs. Thus, for example, we can discuss the implications of amino acid substitutions (as occurs in hemoglobin in sickle-cell anemia) for the folding of the chain into its native conformation. Many diseases, like sickle cell anemia, are now related to amino acid substitutions, and attendant changes in conformation, in various critical proteins.

An intriguing line of research is being conducted which promises to throw additional light on many carbohydrate-containing entities with complex, specific structures and correspondingly specific biological activities. As examples we may cite the blood group substances, a sizeable group of

antibiotics, and the many proteins which carry carbohydrate moieties. The carbohydrate parts of these materials are oligosaccharidic in nature (up to, say, 15 sugar residues). The synthesis of saccharides of these types, with the identity, order, and mode of linkage of the sugar units chosen at will, is potentially highly rewarding. Among other things, one can envision the designers of synthetic proteins frequently wishing to build carbohydrate into their products to give them just the right characteristics.

The application of the highly sophisticated methods employed in temperature jump kinetics has been extended to the study of natural biological as well as artificial membranes. While interpretation of observed data is still speculative, we may obtain new insights on the action of various chemicals on biological membranes. With such a goal in mind it has recently been observed that cyclic polyethers, caffeine, and nicotine when added to vesicle suspensions alter the observed relaxation times. Similar experiments are underway with acetylcholine, strychnine, pilocarpine nitrate, and other chemicals known to alter transmission of nervous system signals.

There is no question that membranes play a key role in biological control. For instance, the binding of enzymes to membranes in mitochondria or to the plasma membrane in bacteria is well known. Similarly, contact inhibition is an as yet unexplained property of normal cells that is absent in cancer cells and is quite likely a consequence of membrane structural changes or transport properties. Thus, kinetic studies of the properties of biological membranes could lead just as readily to an increased understanding of cancer as to a better understanding of how drugs affect transmission of nerve impulses.

Phytol, a decomposition product of chlorophyll, when incorporated into membrane lipids by humans lacking the ability to metabolize this alcohol causes muscle and nerve degeneration. The symptoms of this disorder are relieved to some extent by eliminating chlorophyll containing foods from the diet of affected persons. Results of this type suggest that new insights to diseases such as multiple sclerosis and muscular distrophy may follow from a better understanding of the properties of membranes.

12 - UROLOGY AND RENAL DISEASES

Projects supported by the NIAMD Urology and Renal Diseases program, continue to be broadly based in both basic and clinical problems of the kidney and genitourinary tract. Since its establishment eight years ago, research activities are predominently in the area of nephrology. Support for urological research continues to show a slow, but steady growth.

Renal Transplantation

Efforts to improve immunosuppression and organ preservation continue to be the primary concerns of the renal transplant field. Kidneys are now uniformly preserved for 24 to 48 hours, with transplantation successfully carried out on a routine basis. The survival of cadaveric grafts over the past year was 74% for one transplant team and their success can be largely attributed to the improved preservation techniques.

Because of the success in renal transplantation from related living donors, another group has extended their criteria for the acceptance of individuals with end stage renal disease. Patients with severe juvenile diabetes that has progressed to irreversible glomerulosclerosis (Kimmelstiel-Wilson Disease) have been excluded in most transplantation programs; however, the University of Minnesota group has successfully transplanted five of these patients. Although in most instances, the patients are taking a few more units of insulin than previously, diabetes management has not been a problem in any of these renal recipients despite the use of prednisone in the usual doses.

Many transplant centers participate in a registry of potential transplant patients for the purpose of making available and making better use of cadaveric kidneys as they become available. The Southeastern Regional Program, which includes centers from Baltimore to Atlanta have had notable success. The group reports that tissue typing is better, immediate function is better and incidence of transplants which never function is lower.

With the increasing success of kidney transplantation, the costs have progressively decreased. At present, third party payments cover the majority of the costs. Consequently, transplantation continues to expand to include people from wider sociological and economic backgrounds.

Dialysis

Even though kidney transplantation is the ultimate goal for treatment of patients with chronic renal failure, hemodialysis continues to play an important role by maintaining the kidney failure patient until he is ready for a transplant operation, or until a suitable donor kidney becomes available. Therefore, it is encumbent upon the research community to continually refine dialysis hardware and techniques. Dr. Scribner's group at the University of Washington continues to receive support for their intensive efforts in this area.

Micropuncture Studies

Micropuncture techniques continue to be extensively employed to study individual tubule response to various ionic abberations in the peritubular and/or intraluminal fluid. Cationic, anionic, amino acid and water transport studies can routinely be employed with this technique. The sites of action of many diuretics have been elucidated with micropuncture studies.

Another method that has gained wide reception in studies of nephron function is the microperfusion of individual isolated rabbit nephrons. One investigator, using this preparation, is attempting to find out what regulates the cell's volume. It is generally accepted that anoxia accompanying periods of tissue ischemia results in cellular swelling. The swelling of capillary endothelium and renal tubular epithelium with subsequent abolition of fluid flow may play a role in the morbidity of cerebrovascular and renal disease following periods of systemic arterial hypotension or thromboembolism. More basically, examination of the cellular swelling process allows one to draw some conclusions about the permeability properties of cell membranes to various ions and molecules and the mechanisms by which cells normally regulate their volume.

Another investigator, one who was primarily responsible for developing the individual perfused nephron technique, continues to utilize this preparation to study mechanisms of fluid transport through intercellular spaces. To fully understand these mechanisms, there is need to learn more about the mechanical properties and permeability characteristics of the different membranes which bound the lateral intercellular compartment. He has reported that the deformability of the urinary surface of collecting duct cells was increased by vasopressin. Furthermore, he found no evidence to implicate cytoplasm as a significant factor in determining the change in cell deformability in response to ADH. Dr. Grantham interpreted these findings as indicating the presence of a barrier at the apical membrane with "contractile" properties. Implicit in this view is the supposition that the increase in deformability and increase in water permeability in response to vasopressin are interrelated.

Urinary Concentrating Ability

An investigator at the Dartmouth Medical School continues to study hereditary urinary concentrating defects in the Brattleboro rat model. He has recently expanded these studies by working with a strain of mice that are also known to have nephrogenic diabetes insipidus. This investigator has been able to characterize the concentrating defects of the hypothalamo-neurohypophysial system in these mice by histological techniques. His findings suggest a direct correlation between the amount of fluid turnover and the degree of hypertrophy of this system, as well as between increased neurohypophysial activity and hypertrophy of the pars intermedia of the pituitary gland. Although this same investigator and his colleagues have reported that chlorpropamide appears to ameliorate hypothalamic diabetes insipidus, by potentiating the antidiuretic effect of ADH, medullary accumulation of urea occurs and hence, an increase in medullary osmolality ensues.

An interesting comparative study has been reported concerning the concentrating ability of genetically related bat species who occupy markedly different environments and have different dietary habits. Studies of this species appear to contradict, at least in part, the hypothesis that urine concentrating ability is dependent on the relative thickness of the renal medulla. A more important parameter for renal concentration that is suggested, is actual medullary volume compared to total kidney volume, coupled with relative medullary cellular efficiency. To elucidate this hypothesis, this investigator plans to measure ATPase activity in conjunction with urea C^{14} and Cl^{36} incorporation in serial slices of medullary tissue from several selected species.

Urolithiasis

Urolithiasis is an important medical problem which accounts for hospitalizing one out of every one thousand United States citizens each year. It is a disease that is often recurrent and can affect persons of all ages; however, in the United States it is in the main a disease of adults. Its medical management, except for certain surgical procedures and antibiotics, has remained essentially unchanged for many years. Progress in the field is hampered by a lack of information about the causes of urolithiasis.

During the last five years some research, involving the physicochemical aspects of urinary stone formation, has been done. The initial results of this research are promising; some important observations have been made. But in the opinion of the Committee on the Genitourinary System of the National Research Council, the current effort is inadequate in view of the complexity and importance of the problems involved. The Committee does not believe that a "crash" research program is indicated, but strongly endorses the encouragement of more collaborative research in which urologists, physical chemists, physicists, and possibly researchers from other disciplines may work together using modern methods and pursuing new research concepts. A bonafide effort is being made to stimulate interest among investigators in scientific disciplines not well represented in the area, and to encourage collaborative work among urologists and other investigators in research on the formation of urinary stones.

To this end, plans for a Conference on Urolithiasis are being finalized. The National Institute of Arthritis and Metabolic Diseases will provide monetary support for this conference which will be held at the Mayo Clinic in October 1971.

Other Research

Considerable attention is currently being focused on an angiotensin-sodium interaction in the central control of body fluid and electrolyte balance. For instance, one laboratory has recently reported that solutions of artificial cerebrospinal fluid, containing various concentrations of angiotensin perfused through the ventriculocisternal system of anesthetized dogs, resulted in significant antidiuretic hormone (ADH) release. These same investigators reported that small intravenous infusions of Pitressin, which

increased the concentration in plasma by as little as 0.5 - 5 microunits per milliliter, inhibited renin secretion by the kidney. They conclude that ADH over its normal physiological range, exerts an inhibitory effect on renin release, possibly via a direct action on the juxtaglomerular apparatus. To summarize their findings, angiotensin (the end result of renin secretion) stimulates ADH release and ADH in turn, inhibits renin release, forming a negative feed back system.

Histochemical and some analytical results suggest that the loss of specific filtration properties of glomerular basement membrane (GBM) leading to proteinuria may be the result of altered chemical composition, molecular configuration, or both. The integrity of this entity is of great concern in the studies of such disease states as uremia and renal failure, nephrotic syndrome and glomerulonephritis. Although the precise mechanisms of glomerular capillary wall injury are unknown in these disease states, available evidence emphasizes the importance of complement mediated injury, polymorphonuclear leukocyte infiltration, and the activity of various lysosomal enzymes and cationic proteins on cell membranes and the GBM.

It has been suggested that abnormalities of ureteral peristalsis may lead to deterioration in renal structure and function. Consequently, a number of studies are under way which are directed to define various physiologic, pharmacologic and anatomic properties of normal and abnormal ureters, and to evaluate methods that would eventually improve ureteral activity. Some of the data obtained from various in vitro and in situ studies are being utilized in the development of an implantable radio-frequency electrical pacemaker that may someday provide long-term stimulation of the hypofunctioning ureter, and thus aid in the preservation of renal function.

To convey the sense of the year's developments this report offers a look at the Institute's educational support programs through bifocals: first a close-up of problems attending selection from approved applications of those to be funded, followed by a look at the long-range problem of projections and their modulation by studies of yield.

The selection problem takes its broad outlines from the Institute's responsibility for a dozen areas, one that fosters continuing questions as to consistency in review and equity in funding. Some modifications introduced this year in the procedures of our training committees and Council offer the prospect of dealing more effectively with these perennial concerns. They are reported here briefly.

Gains in the quest for consistency have come from a combination of (1) making discussion of the priority score a part of the review, (2) encouraging the submission of brief comments with individual scores, especially where these depart from values discussed, and (3) reporting individual scores with comments in the Council summary. These measures appear to help the silent minority find a voice; they fail to come to grips with the tendency to confuse a "good" priority with "priority to pay". To deal with the latter, consideration is being given by the Institute to modification of present training committee structure from specialty lines toward multi-categorical combinations.

The quest for equity in fund distribution has been advanced by reserving to one of the three Council meetings (the November meeting) the decisions on training grant funding that were formerly distributed among all three. Obliged by size of the work load to continue spread of initial review over the customary 3 cycles, Council actions are held fluid until the total demand is clearly in view. The initial review group (i.e. the training committee) is given an opportunity to examine and to comment on the rank order of the entire group as determined by priority score, these comments being transmitted to Council.

As an outgrowth of the procedures just described, the Council this year went beyond the usual step of determining an order of payment, and recommended a minimum group to be awarded. By this step a significant gain has been achieved in coping with two of the most difficult determinations we must make in working with a declining effective budget: the rate of decline in the number of centers supported, and the amount allocated to each. The step taken here allows decision on the former to modulate the latter.

The projection problem, the other main topic considered in this report, takes its main outlines from the binge-like character of our efforts---rare 10-year forecasts and, even rarer, 10-year surveys---and an attendant inexperience in making these efforts an integral part of our work. A survey of our evolving practices in this endeavor was presented as a staff report to our Advisory Council, a copy of which is appended. Some follow-through is reported here.

First, the survey of trainees and fellows terminating in 1969 is now well under way, the roster compiled (approximately 500), the mailings completed in May, and replies coming in. Collation of these will be carried out this summer, for subsequent review with training committees and Council.

A slower start has been made on the search for measures of unmet need. One clear lead comes with the renewed self-study initiated among medical subspecialties in the wake of the recent decision to replace certification in internal medicine by certification at the subspecialty level. The hematologists, for example, working in the framework of their professional society, have embarked on a survey of available training opportunities for their subspecialty, looking to development of measures of need for this training and opportunities available to those trained. The rheumatologists have made tentative steps in the same direction. The dermatologists have just completed a survey of the impact of training grant support on the growth of their field. The Institute's Gastroenterology and Nutrition Training Committee has continued its critical inquiry into current support in relation to need, leading to recommendations of exploring the contribution of new support instruments to faculty development. Continuing the trend initiated with the earlier self-studies in dermatology and orthopedics, these efforts are noteworthy for breadth that extends beyond the selective NIH concern with investigation and faculty, providing thereby a valuable context for projecting the NIH-supported component.

Translation of this endeavor into a framework for planning the institute's educational support is clearly a long-range stepwise process.

Appendix: Elements of Planning for the 70's (A staff report on training presented at the November 1970 meeting of the NIAMD Council.)

NIAMD Education Support: Elements of Planning for the 70's

I. Inheritance from the 60's

A. Projection at that time was based on self-assessment of two kinds:

1. Utilizing responses to a questionnaire sent to department chairmen, Dr. Robert Williams prepared in 1958 a projection of subspecialty staffing needs by 1970.

2. Utilizing responses to questionnaires sent by the several Institute training committees to program directors, Dr. Marjorie Wilson prepared in 1963 a projection of training grant growth that could meet projected departmental staffing needs.

Result: Growth projection for Institute training programs in the 60's determined by aggregate departmental goals and training opportunities.

B. Measures of accomplishment for comparison with projection developed only gradually.

1. A survey of training program yield was conducted as part of the 1963 survey.

2. Beginning in 1964, at Council's request, tabulation of individual career choices of stipendiary trainees was made an integral part of renewal review.

3. In one area--gastroenterology--Institute staff prepared in 1967 a 10-year survey of training program yield.

4. Development from these of an Institute-wide perspective has utilized data of two kinds.

a. Trainee career choices reported for renewal review were used as a basis for reports in 1964 and 1969.

b. Follow-up information is being solicited from fellows as well as trainees on completing their training, starting with calendar year 1969 "graduates."

Result: - Recognition of systematic "blind spots" inherent in the several approaches.
- Downward revision of early estimates for yield of full-time faculty from 40% to 25% of trainees.
- Recognition of part-time faculty yield as deserving of special study: possibly equal in numbers to full-time faculty, but difficult to define and report.
- Recognition of limitations to effectiveness of Institute training support inherent in late start and short-term

support for the individual, in subspecialty framework for support of the setting; response to this: introduction of training support modified to encompass more nearly the full range of graduate (even some undergraduate) experience requisite for investigator training, and to provide support for a more comprehensive segment of the school's training resources ("Experimental Training Program").

C. Changing picture of institutional need for non-stipendiary components as provided by training grant support:

1. Divisional activities have grown more diversified, and their support patterns more complex.

2. Over the past five years, training grant budgets have shown a consistent trend toward increased support for professional and ancillary staff.

3. Change in grants policy within that same interval has resulted in loss of any required connection between individuals or positions considered at review and those actually supported.

Result: Mounting difficulty in relating proposed support to need, unless of a global kind; increasing reliance on formula.

II. Planning for the 70's---getting to first base

A. The Institutes were asked to prepare, during the summer of 1970, a 5-year projection for training and fellowships support, one based on stipulated estimates of national need, in the following terms:

1. "Suppose growth of full-time faculty continues recent trends (50% increase in 5 years); or increases at one-half that rate, with subsequent leveling off."

2. "Suppose research dollars to show annual increments in the 2-8% range."

B. Implementing procedure adopted for this exercise: introduce temporary simplifications, and explore available information sources.

1. Simplifications: pooling of training and fellowships of all program areas.

2. Initial separations in accordance with stipulations:

a. A component whose growth would follow the "research dollars" pattern (IIA2 above), one comprising RCA, RCDA, and post-Ph.D. fellowship support.

b. A component whose growth would follow the "faculty" pattern (IIA1 above), one comprising mainly training grants and post-M.D. fellowship.

3. Information sources:

a. Recent trends in faculty growth: JAMA education number provides figures in terms of internal medicine, dermatology, orthopedics and urology.

b. Estimation of fraction of "Internal Medicine" faculty that comes within areas of Institute responsibility: 40% by 1958 survey (IA1 above); 50% by listing in Spring issue of Clinical Research of faculty positions available.

c. Estimation of recent Institute contributions to national need: from training grants, aggregate yield data (IB4a above); from fellowships, contemporary data now being sought.

Result: The initial steps reported here are valuable less for numbers thus generated, and more for the definition given to areas where cooperative effort with the field, in the 70's as in the 60's would make for greater validity of estimated need.

III. To get beyond first base: Projection of need for the several Institute areas; contribution from consultant groups in procuring and incorporating such data into Institute planning.

A. Training Committees: Responsible for review of both training grants and fellowship applications, these committees offer a favorable setting for survey of the relative contribution to manpower needs from training grants and fellowships. Representing to the Institute the interest of their respective fields, they may assist in obtaining estimates of corresponding projected manpower need.

1. Fellowship: As of 1971 the committee's responsibility in fellowship review changes: the panels formerly providing initial review analagous to that provided by the study section, will no longer provide review. At the same time the committees will be introduced to first findings from fellowship follow-up. Areas deserving of study include post-M.D./post-Ph.D. distribution; fellowships to work with faculty supported by training grants: whether grants now active, recently lost or in planning stages.

2. Training grants: As with fellowship applications, the committee's primary training grant responsibility rests with the individual review. In keeping with stated plans, however, a trial was made this fall of following that review with a survey of the year's review as Council must consider it. This step prompted discussion of a variable range of topics, including the following:

a. The rank order as obtained by customary scoring: comment by members during the meeting and by letter; opposing considerations of critical evaluation and competition for funding.

b. The problem of review for applications of committee members: ranking from both parent and ad hoc committees.

c. Finding funds for approvals: consideration of options in working within the competing group of applications, e.g. if training committees could be advised of available dollars, they might work out a revised distribution among the approvals.

d. Extension to non-competing applications: the training committees might attempt a "mid-commitment" assessment of support in relation to productivity.

3. Projection of area needs: self-assessment by professional groups.

a. In 1967 a group sponsored by the American Academy of Dermatologists undertook a survey on a national scale of present resources and projected needs of their field, including consideration of manpower needs for service, teaching and investigation.

b. The orthopedic surgeons and urologists have embarked on comparable studies.

c. A role for training committees in extending this sort of study to all areas of Institute interest.

d. A complementing role: continuing survey of actual Institute contribution to these needs, utilizing follow-up data.

Emerging idea: Application review and inquiry into national need could be mutually reinforcing responsibilities.

B. Council

1. Chief concern has been with training grants. Institute educational support through fellowships and Career Development Awards has been reported regularly with the aid of summary tables.

2. Decisions on training grant funding.

a. Point of departure has been the interdigitated priority list, which brings together recommendations from the Institute's seven training committees (but see IIIA2a above).

b. In the early 60's, Council deliberations could usefully focus on individual applications, characteristically on questions of likely productivity to be decided entirely in context of

the individual application.

c. By the late 60's, the reach of Institute funding had effected a shift of focus to choices among grants of assured productivity, choices to be resolved by reference to a context extending beyond the individual application.

3. Recent Council discussion has pointed up the need for looking beyond enumeration of training grants to inquire on a broader basis what level of support may be appropriate to the several Institute areas. Still keeping that broadening confined within the domain of Institute support, the following deserve consideration.

a. Take numbers of training positions rather than grants as main concern; develop pragmatic guides for arriving at these by a combination of training grants and fellowships.

b. Take into account support of comparable individuals through research grants.

4. Looking beyond the confines of AM extramural support, account may be taken of contribution to research and teaching manpower in areas of Institute interest from the following:

a. AM intramural programs.

b. Other federal sources, e.g. VA.

c. Private sources---e.g. Arthritis Foundation fellowships.

5. Council action on individual renewal applications among the "Experimental Training" group (see IB above) could well be accompanied by a look at its relation to the total Institute picture. Consideration of modification in emphasis (e.g. developing departments) or in duration may also be desirable.

ANNUAL REPORT OF OPERATIONS BRANCH, NIAMD-EP

This Branch is organized into two Sections; (1) Administrative Services Section (2) Operations Section.

Administrative Services Section

Effective administration can contribute to the success of an organization in attaining its goal. The Administrative Services Section has responsibility for matters dealing with people and their money. Its functions include:

(1) Time and Leave Records for all EP staff are maintained accurately.

(2) Staff travel orders and reimbursement vouchers covering an average of 30 to 35 trips were processed each month.

(3) Council travel orders, travel requests, reimbursement vouchers, hotel accommodations, and requests for payment of consultant fees for each of twelve members for three meetings plus a number of site visits were handled by this office.

(4) Training committee members made 400 trips to meetings and/or project site visits at a cost of about $75,000. Requests for reimbursement covering travel and consultant fees were received and processed by this Section and paid from the Chairman's Grant which is managed in this office.

(5) Personnel Actions for recruitment, promotions, separations, reassignments, etc., are prepared in this Section. During the past year the Administrative Assistant in this section became a member of the internal "personnel review committee" to aid in the orderly follow-up of the committee actions.

(6) Other services have been provided, such as, general housekeeping, procurement, space planning, etc.

The major problem in this Section is a severe shortage of personnel. A minimum of one but preferrably two additional full time people are needed.

Operations Section

During the past year, NIAMD-EP had $80,735,000 for Research Grants, $15,072,000 for Training, and $5,740,000 for Fellowships. The Operations Section processed applications, summary statements, encumbrance lists, award statements, activations notices, termination notices, correspondence relating to all phases, and maintained official files on the applications utilizing all available funds.

The workload in this Section is greater than indicated by the number of applications paid because only 25% of the competing applications are actually funded; the other 75% require the same amount of work through the completion of the review process.

Inadequate filing space is a major problem. This Institute has elected to retain all grant records since the beginning of the program to aid in program analysis and operation. Recently a decision was made to dispose of the files in accordance with the policy established by the Records Management Manual which will reduce somewhat the need for additional record storage space.

Despite shortage of personnel in this Section, all essential work was completed.

ANNUAL REPORT OF GRANTS MANAGEMENT BRANCH, NIAMD-EP

During the year, no clear-cut additional duties evolved in the branch, but assimilation of the duties transferred last year to the GMB from Operations continued. In the main, this involved the areas of fiscal management and the review and clearance of changes to awards via the "901" system.

As has occurred for past years, the appropriation bill was not passed until well into the fiscal year. This caused our operating allowance to be doled out in equal monthly segments for a large portion of the year. Because our research grant begin-dates are not distributed evenly throughout the year, we were in the position several times of being ready to make awards, but having no funds with which to pay them. This difficulty was partially alleviated later in the year by our special request to receive an allowance larger than one-twelfth of our total budget for research grants. Another problem associated with the lateness of the appropriation bill and the "control of expenditures" was the hold placed on the awarding of Type 1 (New) research grants. This situation prevailed for several months early in the year.

The negotiation of awards between the Institute and the Principal Investigator has been a smoother process this year. Nonetheless, it still has caused substantial slowdowns in the processing of awards due to such factors as the need for submission of revised budgets. The smoother handling of negotiations resulted from a decision reached by the Institute early in the year to aim for a specific percent negotiation reduction throughout the year.

During the year the Department dropped the "Kelly" list requirement for the clearance of competing grants prior to awarding. This action has simplified the awarding process. The implementation of a new policy on "Protection of Human Subjects" for awards beginning on and after January 1, 1971 has added extra detail to the system.

The personnel situation in the Branch has deteriorated slightly during the year from its previous marginal status. One specialist was lost during the year. A replacement from the Management Intern Program was recruited to work in two separate offices within EP, so this compensated only partly for the loss. Thus, the Branch still needs another grants specialist and/or fiscal management officer.

The Branch has managed to function for yet another year without a full-time Chief of the operation. The Acting Chief has not been able to devote the necessary time to this area except to cheer on its effective staff to continue doing an outstanding job and to bear the burden of an increased work load which has been developing. It is important to note that one of the senior staff members has moved to the Cancer Institute. Because of severe limitations imposed by the personnel ceiling, we have been unable to recruit a successor to her.

The "Central Nervous System" role of the Branch is repeatedly demonstrated by the many special requests frequently made of it to "pull" from our stored information evidences for activities supported in special fields. This aids in defining common and overlapping interests and support frequently shared with other Institutes. The question, "What is your Institute supporting in the way of grants relating to bettering life for children below the age of five?" is an example. Others are; "What is your Institute's support to genetic metabolic disease?" and, "What do you support bearing upon the problem of sickle cell anemia?" While these special questions pose more interesting problems than the routine runs made for recurring information and management purposes, their increased frequency and detail are taxing our abilities to get the required work done on the necessary time schedules. This situation has also made it more difficult for staff to continue with educational opportunities, although this effort has continued at a reduced level.

The Branch is even more dependent upon the Central Computer Facility now that it is completely converted to a magnetic tape operation. The Branch's operation is complicated by any changes instituted in that Facility which affect NIAMD data. For instance, a number of our programs previously working well for our needs, have had to be rewritten in new computer language owing to changes in central operation procedures. We have had more reprogramming than we anticipated. Upon occasion the hazard of the computer being "down" has delayed certain outputs of information desperately needed. However, we must live with this situation.

Our Scientific Coding Section has been more active than in the past in spite of a small drop-off in the total number of research grant applications received. With considerably wider overlap of programs being evident at NIH (as AM with HE, HD, NS, CA, GM, AI, etc.), this group has been giving increased surveillance to appropriateness of Institute assignment of our applications. A few special requests from the Associate Director for NIAMD-EP program analysis have required the scientific coding of training grants and fellowships (which has not yet been instituted on a regular basis due to present large work loads coupled with restricted number of staff). Performance of other important functions further limits this Branch's ability to perform fully its potential (e.g., one of the Section's members has been very active and made real contributions during the year to minority group committee activity).

This Branch is responsible for the tables which follow indicating the actual magnitude of NIAMD-EP operations for the completed 1970 fiscal year and the estimates as of now for 1971.

Table I

National Institute of Arthritis and Metabolic Diseases

Support of Research Grants

Program Area		Actual		Estimated	
		July 1, 1969 - June 30, 1970		July 1, 1970 - June 30, 1971	
Number	Name	No. Grants	$ Encumbered	No. Grants	$ Encumbered
01	Arthritis	189	$ 7,643,738	169	$ 8,070,000
02	Dermatology	50	1,826,849	49	2,057,000
03	Diabetes	157	5,366,308	132	5,050,000
04	Endocrinology	322	11,178,977	276	11,300,000
05	Gastroenterology	248	9,097,619	228	9,183,000
06	Hematology	191	6,249,402	184	7,035,000
07	Metabolism	518	17,260,567	486	18,300,000
08	Nutrition	152	4,430,630	141	4,848,000
09	Orthopedics	90	3,443,623	84	3,208,000
10	Physical Biology & Related Areas	109	3,091,504	104	3,353,000
12	Urology & Renal Diseases	203	7,579,227	200	8,331,000
	Totals*	2,229	$77,168,444	2,053	$80,735,000

* do not include US-JAPAN

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Table II

National Institute of Arthritis and Metabolic Diseases

Support of Graduate Training Grants

Program Area		Actual		Estimated	
		July 1, 1969 - June 30, 1970		July 1, 1970 - June 30, 1971	
Number	Name	No. Grants	$ Encumbered	No. Grants	$ Encumbered
01	Arthritis	29	$ 1,271,623	29	$ 1,411,000
02	Dermatology	32	1,604,149	23	1,364,000
03	Diabetes	21	891,057	19	848,000
04	Endocrinology	47	2,307,451	42	2,119,000
05	Gastroenterology	40	2,087,255	38	2,124,000
06	Hematology	37	2,150,338	36	2,107,000
07	Metabolism	30	1,429,404	29	1,371,000
08	Nutrition	11	469,680	11	576,000
09	Orthopedics	15	530,762	15	552,000
10	Physical Biology & Related Areas	6	845,958	7	1,060,000
12	Urology & Renal Diseases	31	1,484,323	31	1,540,000
	Totals	299	$15,072,000	280	$15,072,000

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Table III

National Institute of Arthritis and Metabolic Diseases

Support of Postdoctoral Fellowship (F2) Awards

Program Area		Actual		Estimated	
		July 1, 1969 - June 30, 1970		July 1, 1970 - June 30, 1971	
Number	Name	No. Grants	$ Encumbered	No. Grants	$ Encumbered
01	Arthritis	8	$ 56,312	8	$ 65,000
02	Dermatology	1	7,875	4	32,000
03	Diabetes	8	64,255	15	124,000
04	Endocrinology	12	103,879	13	97,000
05	Gastroenterology	4	36,412	4	37,000
06	Hematology	5	42,776	6	57,000
07	Metabolism	51	397,839	49	400,000
08	Nutrition	6	51,278	5	49,000
09	Orthopedics	1	10,841	4	33,000
10	Physical Biology & Related Areas	12	94,295	6	46,000
12	Urology & Renal Diseases	4	37,890	10	74,000
	Totals	112	$903,652	124	$1,014,000

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Table IV

National Institute of Arthritis and Metabolic Diseases

Support of Special Fellowship (F3) Awards

Program Area		Actual		Estimated	
		July 1, 1969 - June 30, 1970		July 1, 1970 - June 30, 1971	
Number	Name	No. Grants	$ Encumbered	No. Grants	$ Encumbered
01	Arthritis	8	$ 104,530	11	$ 134,000
02	Dermatology	5	53,307	5	64,000
03	Diabetes	9	104,157	7	74,000
04	Endocrinology	10	114,170	12	136,000
05	Gastroenterology	12	138,895	6	83,000
06	Hematology	9	109,744	9	123,000
07	Metabolism	8	88,297	12	145,000
08	Nutrition	4	48,767	1	19,000
09	Orthopedics	2	15,643	6	67,000
10	Physical Biology & Related Areas	4	34,690	0	-----
12	Urology & Renal Diseases	18	217,755	27	329,000
	Totals	89	$1,029,955	96	$1,174,000

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Table V

National Institute of Arthritis and Metabolic Diseases

Support of K3-K4 Research Career Development Awards

Program Area		Actual		Estimated	
		July 1, 1969 - June 30, 1970		July 1, 1970 - June 30, 1971	
Number	Name	No. Grants	$ Encumbered	No. Grants	$ Encumbered
01	Arthritis	10	$ 239,368	11	$ 258,000
02	Dermatology	4	88,049	4	96,000
03	Diabetes	11	258,427	11	266,000
04	Endocrinology	22	567,749	19	482,000
05	Gastroenterology	16	393,176	16	405,000
06	Hematology	20	535,519	18	471,000
07	Metabolism	14	287,168	17	370,000
08	Nutrition	5	125,708	4	100,000
09	Orthopedics	--	-------	1	25,000
10	Physical Biology & Related Areas	2	40,054	4	84,000
12	Urology & Renal Diseases	9	215,459	9	216,000
	Totals	113	$2,750,677	114	$2,773,000

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Table VI

National Institute of Arthritis and Metabolic Diseases

Support of K6 Research Career Awards

Program Area		Actual		Estimated	
		July 1, 1969 - June 30, 1970		July 1, 1970 - June 30, 1971	
Number	Name	No. Grants	$ Encumbered	No. Grants	$ Encumbered
01	Arthritis	2	$ 58,453	2	$ 56,000
02	Dermatology	---	-----	---	-----
03	Diabetes	1	30,645	1	28,000
04	Endocrinology	12	356,489	12	350,000
05	Gastroenterology	---	-----	---	-----
06	Hematology	1	30,510	1	31,000
07	Metabolism	6	174,857	6	174,000
08	Nutrition	3	86,012	3	84,000
09	Orthopedics	---	-----	---	-----
10	Physical Biology & Related Areas	---	-----	---	-----
12	Urology & Renal Diseases	2	60,743	2	56,000
	Totals	27	$797,709	27	$779,000

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OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS AND SCIENTIFIC COMMUNICATION

OFFICE OF SCIENTIFIC COMMUNICATION

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The Office of Scientific Communications is charged with the general programming and planning of communication activities concerned with the various scientific areas under the purview of the NIAMD. The present activities can be roughly divided into four main categories:

1. Current awareness literature services;
2. Retrospective literature studies;
3. Conferences and workshops; and
4. Other aspects of communication, such as the production of motion pictures, thesaurus development, etc.

Current awareness literature services are provided by the Institute in several specialized areas. There are three publications which provide subject organized listings of literature citations and one publication which expands to include abstracts.

ARTHRITIS AND RHEUMATIC DISEASES ABSTRACTS, the oldest of our publications, with the availability of Excerpta Medica's ARTHRITIS AND RHEUMATISM in this country, will no longer be published after the sixth volume. GASTROENTEROLOGY ABSTRACTS AND CITATIONS, now in its sixth volume, is intended to provide abstracts and literature citations in all aspects of gastroenterology, biochemical or clinical.

The ARTIFICIAL KIDNEY BIBLIOGRAPHY (fifth volume), ENDOCRINOLOGY INDEX (fourth volume), and DIABETES LITERATURE INDEX (sixth volume), all contain citations to current world-wide literature which have been included by the National Library of Medicine in their Medical Literature Analysis and Retrieval System (MEDLARS). In order to serve those who are interested in highly specific areas, we have cooperated with the National Library of Medicine and four universities--Minnesota, Rochester, Case Western Reserve, and Vanderbilt--to produce structured, hierarchically-arranged indexes which function as in-depth current-awareness tools for easy review of particular subjects. The standardized terminology used in MEDLARS makes these publications invaluable for retrospective literature searching.

Conferences and workshops which are sponsored by NIAMD are planned and organized by the Scientific Communications Office. In addition to the organization, which usually includes physical arrangements as well as program planning with the conference chairman, the publication of the proceedings also falls under the auspices of the Scientific Communications Office. This year one conference and two workshops were sponsored by the NIAMD. The conference was the Fourth Annual Contractors' Conference of the Artificial Kidney-Chronic Uremia Program, and the Workshops were: Workshop on the Use of Gastrointestinal Absorbents in Uremia and Workshop on Behavioral Bioassays in Uremia.

A film, "The Conservative Management of Chronic Kidney Insufficiency," which was completed last year and intended for distribution to medical schools,

hospitals, and local medical societies, for medical staff instruction in an area about which little knowledge exists in the practicing community, is now being distributed by the HEW Audio Visual Center, Atlanta, and by the National Kidney Foundation.

The development of thesauri is particularly important to the Scientific Communications Office. Work in this area is done under contract as well as "in-house." These thesauri are very important in helping to maintain consistent and thorough indexing in secondary publications which are done under contract, where contractors may change from year to year. When published they help to bring consistencies to the field in which they are prepared.

A GUIDE TO NUTRITION TERMINOLOGY, a project co-sponsored by the National Institute of Child Health and Human Development, under contract with Vanderbilt University, was published and wide distribution to the nutrition community is being effected through limited free distribution by the two Institutes and sale by Superintendent of Documents, Government Printing Office. Our staff is also working with the National Library of Medicine to improve MEDLARS' vocabulary in subject areas that involve our publications. This enables us to use the most current nomenclature in our indexes.

In addition, numerous other activities which bear on scientist-to-scientist communication as it affects the Institute are dealt with frequently. Areas where further emphasis should possibly be placed are continually in review.

Project Title: ARTHRITIS AND RHEUMATIC DISEASES ABSTRACTS

Contractor: Scientific Literature Corporation

Project Officer: Dr. Keatha K. Krueger

Other Professional Personnel: Dr. Richard Colletti

Man Years: Total: 0.7
Professional: 0.2
Others: 0.50

Project Description:

ARTHRITIS AND RHEUMATIC DISEASES ABSTRACTS was a monthly current-awareness journal published by the Office of Scientific Communication, NIAMD. The sixth volume has been completed and contained, each month, approximately 250 abstracts of the current world literature pertaining to this field. The classification of the abstracts followed a modification of the proposal of the Nomenclature and Classification Committee of the American Rheumatism Association. Detailed subject and author indexes were provided.

With the completion of Volume VI, NIAMD will no longer continue to publish ARTHRITIS AND RHEUMATIC DISEASES ABSTRACTS. As agencies of the Federal Government may not publish secondary publications in fields where such a publication is commercially available, and EXCERPTA MEDICA's ARTHRITIS AND RHEUMATISM in recent years has become commercially available in the United States and Canada, the production of our publication is no longer justified.

ARTHRITIS AND RHEUMATIC DISEASES ABSTRACTS was available, free upon request, to qualified investigators with NIH grants or contracts in the field of arthritis, as well as to other government agencies with responsibilities in this area, medical school and hospital libraries. In addition to the free distribution of this publication, it was for sale at a nominal price by the Superintendent of Documents, Government Printing Office.

Project Title: ARTIFICIAL KIDNEY BIBLIOGRAPHY

Professional Personnel: Miss N. Victoria Ragin
Dr. Keatha K. Krueger
Dr. L. C. Terry
Mrs. Billie Mackey

Man Years: Total 0.8
Professional: 0.5
Other: 0.3

Project Description:

The ARTIFICIAL KIDNEY BIBLIOGRAPHY, now in its fifth volume, is a quarterly recurring bibliography on kidney failure, artificial kidneys and kidney transplantation, which is being produced with the aid of National Library of Medicine's MEDLARS (Medical Literature Analysis and Retrieval System) by the Scientific Communication Office, NIAMD. It contains about 500 citations per issue with the citations arranged hierarchically to enable similar subjects to appear together. An author index is included with each issue.

The ARTIFICIAL KIDNEY BIBLIOGRAPHY is made available without cost to National Institutes of Health grantees or contractors who are involved with work related to artificial kidneys, as well as to other government agencies with responsibilities in the area, medical school and hospital libraries. In addition to the free distribution, it is for sale by the Superintendent of Documents, Government Printing Office at a nominal price.

Plans are being made to expand this bibliography to include other aspects of relevance to research on artificial kidneys, such as kidney diseases and nephrology. An editorial advisory committee has been assembled and preliminary MEDLARS searches have been developed.

Project Title: DIABETES LITERATURE INDEX

Contract Number: Ph43-67-663

Amount: $78,145 (April, 1971 - March, 1972)

Contractor: University of Minnesota

Project Officer: Dr. Keatha K. Krueger

Other Professional Personnel: Miss N. Victoria Ragin
Mrs. Billie Mackey

Man Years: Total: .70
Professional: .32
Others: .38

Project Description:

DIABETES LITERATURE INDEX, a monthly current-awareness publication of the NIAMD (now in its sixth volume) was brought about through the cooperative efforts of NIAMD, NLM, the American Diabetes Association, and three universities (University of Minnesota, University of Rochester, and Case Western Reserve University), as well as a number of individual scientists.

Each month MEDLARS magnetic tapes, which contain all of the current biomedical literature citations used for the preparation of the NLM's INDEX MEDICUS, are used for identification of all diabetes-related bibliographic citations. Through 1970 the DIABETES LITERATURE INDEX has consisted of a keyword in title index and an author index resulting from a computer printout. Starting in 1971, the magnetic tape version of these indexes (prepared at the University of Minnesota) is used to generate copy for offset printing by the Government Printing Office on their Linotron. There are now three formats consisting of a hierarchical subject index of core diabetes literature, an abbreviated keyword in title index of all diabetes-related literature, and an author index including index terms along with the citations (for the first author only). Printing the citations under a preconceived hierarchical structure, which groups related subjects, makes it easier for the user to scan his particular interests in one section.

DIABETES LITERATURE INDEX is published monthly by the Office of Scientific Communication, NIAMD, and is available, free upon request, to qualified individuals with NIH grants or contracts who are working in the field of diabetes research, as well as to other government agencies with responsibilities in this area, medical school and hospital libraries. In addition to the free distribution of this publication, it is for sale at a nominal price by the Superintendent of Documents, Government Printing Office.

Project Title: ENDOCRINOLOGY INDEX

Professional Personnel: Miss N. Victoria Ragin
Dr. Keatha K. Krueger

Man Years: Total: 0.75
Professional: 0.50
Others: 0.25

Project Description:

The ENDOCRINOLOGY INDEX, now in its fourth volume, is a bimonthly publication of the NIAMD, which is produced by the National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS). Each issue contains about 4,000 citations of the current world literature in endocrinology. The citations are grouped in eight categories (pituitary, thyroid, etc.) and each category has a preconceived hierarchical organization of subject headings. There is also a "keyword" abstract for each citation which lists all of the index terms assigned to the original article in the author section. Author and subject indexes appear in each issue.

During this past year, arrangement was made with National Library of Medicine's MEDLARS indexers and the Editorial Office of the Endocrine Society for two endocrinology journals, ENDOCRINOLOGY and JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM to be indexed directly from manuscripts by NLM's indexers resulting in faster input into MEDLARS, and consequently, into ENDOCRINOLOGY INDEX.

The ENDOCRINOLOGY INDEX is made available, free upon request, to NIH grantees or contractors in the field of endocrinology and to medical school and hospital libraries. In addition to the free distribution of this publication, it is for sale at a nominal price by the Superintendent of Documents, Government Printing Office.

Project Title: GASTROENTEROLOGY ABSTRACTS AND CITATIONS

Contract Number: NIH 71-2071

Amount: $59,850 (January, 1971 - April, 1972)

Contractor: Scientific Literature Corporation

Project Officer: Dr. Keatha K. Krueger

Other Professional Personnel: Dr. Peter Donshik
Dr. Eric J. Maybach

Man Years: Total: 1.8
Professional: .85
Others: .95

Project Description:

In January 1966 the NIAMD began publication of a current-awareness journal for gastroenterologists. GASTROENTEROLOGY ABSTRACTS AND CITATIONS, now in its sixth volume, appears monthly. Each issue contains approximately 350 abstracts of the most significant gastroenterological literature published in the world and approximately 750 citations covering other relevant gastroenterological literature. The abstracts and citations are divided into three main categories, Pre-clinical Sciences, Diagnostic Procedures and Gastrointestinal Diseases. These categories are subdivided into more specific groupings. Each issue contains a subject and author index and these are cumulated annually.

GASTROENTEROLOGY ABSTRACTS AND CITATIONS is available, free upon request, to qualified investigators with NIH grants or contracts in the field of gastroenterology, as well as to other government agencies with responsibilities in this area, medical school and hospital libraries. In addition to the free distribution of this publication, it is for sale at a nominal price by the Superintendent of Documents, Government Printing Office.

Project Title: Guide to Nutrition Terminology

Contract Number: PH43-67-1449

Amount: Extended to September 1970 without added funds.

Contractor: Vanderbilt University (Dr. E. Neige Todhunter, Principal Investigator)

Project Officer: Dr. Keatha K. Krueger

Co-sponsoring Agency: NICHD

Man Years: Total: 0.2
Professional: 0.2
Other: 0

Project Description:

A GUIDE TO NUTRITION TERMINOLOGY FOR INDEXING AND RETRIEVAL was previously evaluated by a limited number of individuals who represented all of the different disciplines included in the field of nutrition. The Guide includes alphabetized and categorized lists of nutrition terms as well as cross referenced related terms.

The Guide was published in July, 1970, and wide distribution to the nutrition community is being effected through limited free distribution by the two Institutes and sale by the Superintendent of Documents, Government Printing Office.

Project Title:	Film on "The Conservative Management of Chronic Renal Insufficiency"
Contractor:	National Kidney Foundation, Inc.
Project Officer:	Dr. Keatha K. Krueger
Man Years:	Total: 0.05 Professional: 0.05 Others: 0

Project Description:

A thirty-minute color film designed for professional audiences dealing with various aspects of conservative management of chronic renal failure which was filmed last year is now available for distribution by the HEW Audio Visual Center, Atlanta, and by the National Kidney Foundation. It is available to medical schools, hospitals, medical societies and other professional groups and individuals. Dr. Louis G. Welt, Professor and Chairman, Department of Medicine, The School of Medicine, University of North Carolina, and Dr. Robert Berliner, Deputy Director for Science, NIH, were consultants to the film production.

Project Title: Artificial Kidney - Chronic Uremia Program
Third Annual Contractors' Conference Proceedings

Professional Personnel: Dr. Keatha K. Krueger
Miss N. Victoria Ragin

Man Years: Total: 0.1
Professional: 0
Others: 0.1

Project Description:

The Third Annual Contractors' Conference was held January 14-16, 1970. About 150 participants who were contractors and their staff consultants to the Artificial Kidney Program, and Institute staff attended. The three-day session involved extensive study of existing contract-funded research and development projects with emphasis on intercommunication of program progress and projected plans for the future.

Proceedings of the conference were compiled and given wide distribution to the community of scientists interested in the field of artificial kidney and chronic uremia.

Project Title:	Artificial Kidney - Chronic Uremia Program Fourth Annual Contractors' Conference and Proceedings
Professional Personnel:	Dr. Thomas M. Valega Dr. Keatha K. Krueger Miss N. Victoria Ragin
Man Years:	Total: 0.4 Professional: 0.2 Others: 0.2

Project Description:

The Fourth Annual Contractors' Conference, held January 20-22, 1971, brought together about 150 contractors' key staff members with consultants to the Program and Institute staff. The three-day session involved extensive study of existing contract-funded research and development projects with emphasis on program progress and projected plans for the future.

The conferees, in both plenary sessions and intensive individual workshops, communicated research results in the main areas of the Artificial Kidney Program: 1) Hardware and Mass Transfer; 2) Cannulas, Membranes, and Northrombogenic Surfaces; and 3) Clinical and Metabolic Studies. A considerable body of detailed and pertinent scientific and technical information was exchanged between the top staff of 65 currently active research and development projects, the Program's consultants and its staff. It is anticipated that this and future conferences will significantly assist attainment of the Artificial Kidney Program's goals--optimum artificial kidney development, improved clinical methodology, and better patient rehabilitation.

The Proceedings of the conference will be published and distributed in June, 1971.

Project Title:	Behavioral Bioassays in Uremia Workshop and and Proceedings
Professional Personnel:	Dr. Richard Colletti Dr. Keatha K. Krueger Miss N. Victoria Ragin Mrs. Billie Mackey
Man Years:	Total: 0.8 Professional: 0.4 Other: 0.4

Project Description:

The Workshop on Behavioral Bioassays in Uremia was held on November 19, 1970 at NIH. The aim of the Workshop was to bring together experts from several disciplines who were interested in relating the knowledge of clinical and experimental psychology, pharmacology, neurology, and nephrology to the problem of behavioral bioassays in uremia, and to elucidate pathogenesis of behavior in uremia. Thirty-five investigators discussed available and potential behavioral bioassays of uremia.

Clinically observable effects on the central nervous system and behavior in man include drowsiness, impaired memory, and failure to sustain attention (all of which may progress to psychosis, convulsions, and coma in severe or untreated disease). As these uremic symptoms show a poor correlation with patients' blood analysis, other methods of determining uremic patients' conditions were sought such as correlation of patients' EEG and time of dialysis, and patients' ability to perform various tests involving alertness, visual perception and eye and hand coordination. Attempts to utilize behavioral technology to develop a behavior bioassay for changes associated with acute renal failure were summarized.

Proceedings of the Workshop have been compiled and will be published in the near future.

Project Title: CBAC Project with DCRT

Professional Personnel: Miss N. Victoria Ragin
Dr. Keatha K. Krueger
Mrs. Billie Mackey

Man Years: Total: 0.1
Professional: 0.05
Other: 0.05

Project Description:

In January, 1971, DCRT conducted sessions to publicize their newly acquired CBAC (Chemical-Biological Activities) computer tapes, and to generate interest among the scientists in using these tapes for bibliographic purposes. Searchable data elements on tape are the abstract, title of article, authors, original journal reference, molecular formulae and Registry Numbers assigned by Chemical Abstracts for each chemical compound. Because of the large number of basic research scientists in NIAMD this Institute was chosen to test the utility and desirability of providing this service on a cost basis NIH-wide. The Scientific Communications Office (cooperating with Dr. J. E. Rall who is coordinating the effort) furnished a list of interested NIAMD scientists to DCRT, helped construct profiles for these scientists, designed an evaluation form, and will help evaluate the responses of the scientists.

Project Title: Conference on Progress in Methods of Bone Mineral Measurement--Proceedings

Professional Personnel: Dr. G. Donald Whedon
Dr. Keatha K. Krueger
Miss N. Victoria Ragin

Man Years: Total: 0.1
Professional: 0.0
Others: 0.1

Project Description:

This conference was held at NIH on February 15-17, 1968 with Dr. G. Donald Whedon, Director, NIAMD, as one of the co-chairmen and organizers of the conference. The purpose of the conference was to evaluate and compare techniques under development to relate bone density and mineral content to the degree of pathology in bone disease and to establish normal ranges of mineral content according to sex and age. The proceedings were published in July, 1970.

Project Title: Symposium on Organ Transplantation--Proceedings

Professional Personnel: Dr. G. Donald Whedon
Mr. Blair Sadler
Dr. Alfred Sadler
Dr. Keatha K. Krueger
Miss N. Victoria Ragin

Man Years: Total: 0.1
Professional: 0.0
Others: 0.1

Project Description:

This conference was held in conjunction with the Maryland Academy of Sciences on May 24, 1969. Human organ transplantation and the many ethical, social, and legal implications involved in these great technical advances were discussed. The conference proceedings, which presented discussions relevant to and an up-to-date summary of the medical aspects of transplantation and the medical-legal concerns they present, were published and distributed to the interested scientific community.

Project Title:	Uremic Toxins Conference Proceedings
Professional Personnel:	Dr. Benjamin T. Burton Dr. Keatha K. Krueger Dr. Eric J. Maybach Miss N. Victoria Ragin
Man Years:	Total: 0.2 Professional: 0.1 Others: 0.1

Project Description:

The conference on uremic toxins was held March 18-20, 1970. The twenty-three speakers, both basic and clinical scientists, explored current research on the complications (hematologic disorders, neurologic disorders, intermediary metabolism disorders and nitrogen and mineral metabolism abnormalities) associated with uremia.

The proceedings were published in the Archives of Internal Medicine in November, 1970. This was the fourth of a series of conferences directly pertaining to the mission of the Artificial Kidney-Chronic Uremia Program of NIAMD.

Project Title:	The Use of Gastrointestinal Absorbents in Uremia Workshop and Proceedings
Professional Personnel:	Dr. Peter Donshik Dr. Keatha K. Krueger Miss N. Victoria Ragin Mrs. Billie Mackey
Man Years:	Total: 0.8 Professional: 0.4 Others: 0.4

Project Description:

The Workshop on the Use of Gastrointestinal Absorbents in Uremia was held on January 19, 1971 at NIH and was attended by twenty-four experts in the fields of nephrology, gastroenterology, chemistry, pharmacology, and engineering to study the problem of using gastrointestinal sorbents as a mode of therapy in uremia.

Four investigators currently holding contracts in the Artificial Kidney-Chronic Uremia Program described their research findings including: a system of microcapsules that were able to bind urea and ammonia in vitro, data (in vitro and in vivo) on microencapsulated and unencapsulated activated charcoal, and in vitro studies on the ability of dialdehyde starch to remove urea and ammonia. A low-volume sorbent-based dialysate regeneration system has been used for periods of six months without evidence of acute or chronic toxic effects. This dialysate regenerating system used one liter of dialysate which was recirculated through sorbent columns composed of zirconium phosphate, carbonated zirconium oxide, and activated charcoal. Demonstration of the effectiveness of these sorbent agents was unsuccessful when the agents were administered orally or rectally to either normal or "uremic" dogs. On the other hand, in vitro and in vivo data was presented that supported the effectiveness of oxidized starch administered orally for the removal of urea and ammonia. In addition, a presentation on the metabolism of urea and ammonia in the gut of normal and uremic individuals was given by a world authority in this area.

The considerable discussion identified areas where there was a scarcity of knowledge, and where further study is needed, which included: physiology of the gastrointestinal tract in uremia, whether toxic substances associated with uremia other than urea would be excreted into the gut, and the interaction of gastrointestinal fluid components and sorbents.

The Workshop, while not offering any concrete answers, was beneficial in delving into those areas that needed further study and in opening new avenues of investigation. Proceedings are being compiled and will be published in the near future.

OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS
AND SCIENTIFIC COMMUNICATION

ARTIFICIAL KIDNEY - CHRONIC UREMIA PROGRAM

Summary Report

Introduction

The Annual Reports for the previous two years provide a detailed background description of the problem of end-stage kidney disease and elaborate on a relatively new therapeutic modality for its treatment, chronic intermittent hemodialysis. In addition, these reports give a detailed description of the planned and centrally directed contract program of research and development organized at the National Institute of Arthritis and Metabolic Diseases in 1966 -- the Artificial Kidney - Chronic Uremia Program -- which is aimed at development of improved and less expensive dialysis hardware and methodologies and at optimal rehabilitation of chronic dialysis patients. The Annual Report for 1968-1969 also has outlined in detail the problems of highest priority to which the Artificial Kidney - Chronic Uremia Program is addressing most of its efforts. This background material is not repeated here but the reader might wish to familiarize himself with it by perusing the previous reports.

Current Activities

The Artificial Kidney - Chronic Uremia Program endeavors to bring about improvements in artificial kidney apparatus and methodologies, to increase their effectiveness, to decrease their initial and operational cost, and to improve the rehabilitation of patients treated with chronic intermittent dialysis. Optimal development of new, better dialysis apparatus and methods is still hampered by incomplete knowledge concerning the specifics of the uremic syndrome. Hence, knowledge gained from research into the fundamental aspect of the toxic nature of uremia and basic and clinical studies into the long-term effects of uremia and of chronic intermittent dialysis are critical to arriving at a reasonable solution to the overall problem. Thus, the Program not only sponsors hardware improvement per se, but stimulates and supports a wide spectrum of research ranging from fundamental studies designed to clarify the toxic nature of uremia and clinical studies on the complications of chronic uremia and maintenance dialysis, to development of improved dialysis on apparatus. During the last year there has been an increasing emphasis on laboratory and clinical studies intended to bring about a greater understanding of the nature of uremia and its major complications.

At present about 70 carefully selected research and development projects are in existence, financed by the Institute's contract funds. These contracts were placed with universities, nonprofit research laboratories and industrial concerns, and constitute a broad-spectrum approach to the problems of highest priority. Several projects each are focused onto the problem areas mentioned above and to related investigations which promise to make the treatment of end-stage kidney disease less expensive and more effective, including evaluation of special protein-restricted diets in the maintenance of uremic patients

for whom chronic dialysis is deemed not feasible or unobtainable, and the maintenance of a national registry of all patients who are currently being maintained with the aid of dialysis.

Accomplishments

Among the achievements of the last year were the development of a new generation of compact artificial kidneys, the so-called "hollow fiber dialyzers." The first of these has been introduced into commercial production after having proven extremely effective and relatively simple and inexpensive in use. Similar compact dialyzers of slightly different design are in the process of development.

Another innovation is a new, easy-to-use, presterilized "envelope dialyzer" which is now produced commercially and which greatly simplifies home dialysis with the conventional Kiil equipment. Another contractor of the Program developed a new, clinically successful method of a closed-loop, automated peritoneal dialysis with permanently implanted access devices to the abdominal cavity.

This system has now been introduced into experimental usage in the home where it promises greater safety and convenience coupled with the greatly reduced cost for the otherwise expensive sterile and pyrogen-free dialysis fluid. As additional favorable experience with this type of system accumulates, it is possible to foresee that automated peritoneal dialysis based on permanently indwelling abdominal catheters will have a place, albeit limited, in the broad picture of end-stage kidney disease care -- particularly in cases where briefer periods of maintenance therapy are foreseen as, for instance, in the case of patients waiting for renal transplantation where a definite living donor is at hand.

Another consideration for a closer look at automated, closed-loop peritoneal dialysis with a permanently indwelling abdominal catheter is the fact that it can be safely carried out in the home after a relatively brief training period and by patients who live alone. This type of dialysis is also indicated in those patients where all available locations for blood vessel shunts have been used up.

Another contract resulted in a significant improvment of the conventional Kiil dialyzer; extended clinical tests during the last year of this new high-performance device give promise that "one shift dialysis" with parallel plate (Kiil type) dialyzers may become a reality. The conventional Kiil type dialyzer is preferred by many clinicians because it appears to result in somewhat better rehabilitation of patients; on the other hand, its use (from 9 to 15 hours per dialysis session) extends beyond a single nursing shift in the hospital. Hospital dialysis within the time limits of a single nursing shift would result in major savings for this currently very expensive method of treatment.

Development of zirconium compounds as selective sorbents for uremic wastes, accomplished under an Institute-supported research contract, has now led to further industrial development of new dialysate delivery systems which use only two liters of dialysate which is being constantly regenerated with the aid of zirconium phosphate, zirconium oxide, and activated charcoal sorbent columns. This new development which is now being clinically tested, makes possible the design of compact, portable, suitcase-sized artificial kidneys.

This drastic reduction of the amount of dialysate required also promises to solve a whole series of problems which have beset dialysis, namely the quality of the available tap water and the preparation of large volumes of dialysate with the aid of proportioning pumps and concentrate solutions. When only two liters of tap water are used per dialysis the vagaries of its mineral composition are less worrisome and it is also within the realm of feasibility to envision the future sale, for home dialysis, of a prepackaged dry mix of dialysate salts to be dissolved in the requisite small amount of distilled or deionized water.

A contractor of the Program at the University of Naples, Italy has reported a "first" -- the use of a novel adsorbent material which can be fed to patients and which specifically removes urea directly from the gastrointestinal tract of the patient, and, indirectly, from his bloodstream. He has now shown in clinical experiments that patients who ingest daily 20 grams of this material in divided doses can reduce the frequency of hemodialysis and peritoneal dialysis considerably. Similarly, addition of this oral sorbent to the selected low protein diet (Giordano-Giovannetti diet) for chronic renal failure greatly improves the clinical status of such patients. Other contractors have demonstrated the possibility of maintaining selected patients with a combination of a special low protein diet and infrequently administered hemodialysis; this again is a development which permits a significant decrease in the cost of maintenance dialysis for such patients.

Future Activities of the Artificial Kidney Program

The Program will continue all promising current research and development efforts and will pursue any new ideas and approaches which appear feasible. The Institute is currently funding a number of studies aimed at the isolation and identification of unknown toxic factors which must be removed from the patients blood, in addition to metabolic wastes and other substances known to accumulate during kidney failure, such as urea, creatinine, uric acid, potassium, hydrogen ion, and water. In this regard the program is actively pursuing the lead of the origins and effects of guanidinosuccinic acidemia and of methylguanidine in uremia. Studies, supported by this program, have shown that both substances are elevated in uremics, and have a pathophysiologic correlate to uremic symptoms. Searches will also be pursued for other toxicants possibly involved in the uremic syndrome. Much of the future success in simplifying the treatment of patients with end-stage kidney disease will depend on developments in these areas. The Program also intends to follow very closely clinical and technological developments

abroad and, when promising, to enlarge upon them for the benefit of United States patients as well. This is particularly pertinent since developments in several Western European countries have been quite promising and their early and active introduction in the United States, and possible refinement (without waiting for the usual delay connected with spontaneous spread of new techniques), could prove highly beneficial to patients in the United States.

Conferences, Publications, and Scientific Communication

In March 1970, the Institute organized and held a scientific workshop on the unknown toxic factors in uremia in an effort to stimulate a scientifically relatively stagnant area in which further progress is essential to breakthroughs in the treatment of end-stage kidney disease. The full proceedings of this conference have been published as the November 1970 issue of Archives of Internal Medicine as well as in the form of a separate monograph. This monograph has been distributed widely (including the entire membership of the American Society of Nephrology) and has received very laudable comment.

In January 1971, the fourth annual review meeting of the Institute's Artificial Kidney - Chronic Uremia Program contractors brought together about 200 contractors' key staff members with consultants to the Program and Institute staff. The three-day session involved extensive study of existing contract-funded research and development projects with emphasis on program progress and projected plans for the future. A considerable body of detailed and pertinent scientific and technical information was exchanged between the top staff of the currently active research and development projects, the Program's consultants and its staff. As in previous years, this material will be published in the form of concise Proceedings to be widely distributed to workers in the relevant fields. The Proceedings of the previous annual conferences have become definitive publications on the state of the art in dialysis and it is anticipated that this and future such conferences will significantly assist attainment of the Artificial Kidney - Chronic Uremia Program's goals: optimal artificial kidney development, improved clinical methodology, and better patient rehabilitation.

The Program has organized a one-day interdisciplinary workshop on the topic of behavioral bioassays in uremia in December 1970, and another one-day interdisciplinary workshop on the topic of intestinal sorbents in uremia in January 1971. In each case a number of invited outside authorities in various relevant disciplines joined Program contractors, consultants and staff in a one-day discussion concerning these new and unexplored fields. The proceedings of both workshops will be printed and widely disseminated to interested investigators and the membership of the American Society of Nephrology.

A 30-minute color film "Diagnosis and Management in Chronic Kidney Disease," for the medical profession, has been produced as part of the Artificial Kidney Program and will be widely distributed to teaching hospitals and county medical societies to advance professional education in chronic kidney disease.

The quarterly publication Artificial Kidney Bibliography is now in the process of being greatly enlarged and broadened in scope and it is expected that within a year or two it will become a regular bimonthly publication of the Institute covering the entire field of kidney disease -- Kidney Disease and Nephrology Index.

Outlook for Kidney Disease and Research and Development

Research conducted and supported by the National Institute of Arthritis and Metabolic Diseases has added much to the understanding and treatment of kidney disease. Investigations such as those discussed in this report have increased our ability to cope with the problems of kidney failure. Additional new research efforts are called for in order that a more widespread and permanent control of kidney disease can be achieved. A meaningful program to reduce kidney disease must include all of the various components -- research, prevention, treatment, education -- available in our armamentarium. Such a total program must be aimed at all primary kidney diseases as well as at end-stage kidney failure. This combination, led by much-needed research, should yield maximal benefits in the number of lives saved today and the deaths prevented in the years to come.

Contractor: Abcor, Inc.

Amount: $148,900

Title: Improved Cannula Materials for Hemodialysis

Objectives:
This contract is designed to develop and explore potential syn thetic materials which will produce an artificial vascular intima and then fabricate a blood compatible hemodialysis cannula system from these materials.

Major Findings:
Work is continuing on the development of artificial intimas. Electro-deposited materials are being utilized in an attempt to make an extremely thin artificial vascular intima.

Proposed Course:
There will be continued efforts at developing the various materials being used for an artificial vascular intima.

Contractor: Abcor, Inc.

Amount: $138,600

Title: Development of Capillary Membrane Dialyzer

Objectives:

To develop an improved artificial kidney utilizing capillary membranes at the dialyzing surface and to clinically test this device.

Major Findings:

The dialyzer has been designed, built and has been in the process of clinical testing. There have been problems with this dialyzer in that recurrent conjunctivitis has been noted. It is thought that this may be related to the formaldhyde in the sterilization procedures of the dialyzer. Other designs of dialysate flow within the kidney are being investigated by the company and its marketing partner. Semi-automated procedures have been developed to provide a production train for treatment of the fibers. Full scale units are able to be fabricated and the clinical testing program awaits further funding at the present time.

Proposed Course:

Should further funding of this project be forthcoming there will be continued clinical testing of the current model capillary dialyzer as well as any future models which are currently being developed.

Contractor: Aerojet-General Corporation

Amount: $185,273

Title: Research and Development of Hemodialysis Membranes

Objectives:

The contractor has been working with the objective of developing membranes for hemodialysis by using membranes of asymmetric cellulose or cellulose derivatives. He has also designed his work so that these membranes can be made cheaply and in large enough quantities for use with present hemodialysis equipment.

Major Findings:

The contractor has successfully prepared asymmetric membranes of cellulose acetate, diethylaminoethyl cellulose acetate (DEAECA), and cellulose acetate hydrogen succinate (CAHS). These membranes have been extensively tested and cellulose acetate has been shown to have somewhat improved transport of sodium chloride, urea, and creatinine with superior transport of uric acid. DEAECA has facilitated transport of acidic compounds while CAHS has facilitated transport of basic compounds. Ultrafiltration rates remain significantly higher than presently available membranes and controllable. Patients have been dialyzed at the Wadsworth Veterans Administration Hospital with the use of the Aerojet asymmetric cellulose acetate membrane. The membrane proved to be the excellent ultrafilter predicted, caused no adverse reactions, and performed generally well. To date two patients have been dialyzed using the cellulose acetate membranes and good results have been achieved.

Proposed Course:

Aerojet will devote its time to perfecting the cellulose acetate membrane clinical trials and scaling up their production of it. They will attempt to have it evaluated at Seattle by Dr. Scribner's group and by Dr. Lavender. They have been asked to discontinue production of CAHS and DEAECA until the actual value of CA as a dialysis membrane has been verified. They have been able to lower the UF rate of CA membranes to 3-4 X that of cuprophane.

Contractor: Albany Medical College of Union University

Amount: $8,600

Title: Electronic EEG Frequency Analysis for Evaluation of Uremia

Objectives:
The contractor's objective is to attempt to devise an electronic system which would enable detailed and sophisticated analyses of the EEG in uremia and to attempt to use it as a tool to predict the severity of the clinical status of the patient.

Major Findings:
The program has developed the proposed electronic system and has demonstrated that even well dialyzed patients have abnormally slow EEGs when studied in this technique. However, in transplanted patients the abnormal slowing of the EEG was resolved in the first week after successful transplant.

Proposed Course:
The investigator plans to apply this technique on patients at other centers to confirm the results he has obtained. Should the results be confirmed, then he expects to simplify the machine so it can be used as way of judging the clinical state of the patient and the success of therapy.

In addition, he will study why the EEG of some dialyzed patients returns to normal while in others it does not.

Contractor: American Hospital Supply

Amount: $85,800

Title: Improved Cannula Development

Objectives:
This is a contract to develop an improved cannula with a flare tip design on the venous side. In addition an artificial type of sleeve pin design will be fabricated and tested *in vivo*.

Major Findings:
Preliminary flow studies indicated that a continuously slowly expanding tip of approximately 6 to 8 degrees taper seems to give a better flow in the cannula than does a more sharply divergent tip. Cannulas of various tip configurations have been developed and fabricated. A subcontract with the University of Washington is in the process of being negotiated for *in vivo* testing of these tip designs.

Proposed Course:
There will be *in vivo* evaluation of the already fabricated venous tips and based on the results of these *in vivo* tests further development of cannula tips will be done.

Contractor: Amicon Corporation

Amount: $141,170

Title: An Improved Hollow-Fiber Hemodialyzer

Objectives:

The new contractor will continue to develop and evaluate a new hollow-fiber hemodialyzer. They will share the *in vitro* and animal testing with Cutter Laboratories as a subcontractor. It should be ready for clinical trials at the end of one year.

Contractor: Argonne National Laboratory

Amount: $75,000

Title: An Adhesive Bonded Small Low Blood Volume Parallel Flow Hemodialyzer

Objectives:

The objectives of this contract are to improve the design of the miniature parallel flow hemodialyzer.

Major Findings:

A small hemodialyzer (22.5 cm long composed of 80 layers of cellophane membranes) was tested. Work on improving the dialyzer design has involved using pleated cellophane bonded to flat cellophane to make planes of membrane instead of using standard cellophane with vexar supports ("Model 7").

Proposed Course:

The major emphasis will be on building and testing a prototype of the "Model 7".

Contractor: AVCO Everett Research Laboratory

Amount: $30,792

Title: Blood Flow Considerations to Minimize Thrombosis in AK Systems

Objectives: The objectives are the following: 1) determine relevant flow parameters to the formation of thrombi; 2) quantitate these parameters for materials relevant to artificial kidneys; 3) assess the significance of these parameters in the design of artificial kidneys.

Major Finding:

Two experimental flow configurations, were studied. Preliminary data indicates that thrombus growth is limited by both the concentration of activated protein and the flow of formed blood elements, platelets and red blood cells, to surface. In a pipe flow configuration the concentration of such formed elements is greatest at the tube entrance and decreases over the length of the tube as the formed elements adhere to the surface.

Proposed Course:

To investigate the events leading to thrombus formation in small tube. They plan to study thrombus growth rates and investigate the effect of increasing shear on thrombus growth.

Contractor: Avco Everett Company

Amount: $75,000

Title: Development of Avcothane Cannulas

Objective:
Utilizing the proprietary substance Avcothane, these people proposed to develop a cannula of this Avcothane as well as to investigate various causes for recurrent thrombosis with the use of cannulas in hemodialysis.

Major Findings:
This is a new contract which has just been initiated.

Contractor: Battelle Memorial Institute

Amount: $95,200

Title: Feasibility of Microcapsulated Detoxicants for Removal of Metabolites Via Ingestion

Objectives:

The overall objectives of this contract are the development of microcapsules capable of irreversibly binding urea, ammonia and other toxic metabolites and the evaluation of these capsules for gut dialysis.

Major Findings:

The Battelle group have found that chemical binders of urea are inadequate and the most rewarding area of investigation is to develop methods to stabilize urease and aspartase and bind these enzymes to polymers which can then be encapsulated. The urea would then react with the urease and aspartase and be converted to ammonia which then could be adsorbed by resins containing sulfonic acid groups which are also encapsulated. The stabilized urease has been encapsulated as well as the resins containing sulfonic acid. Their microencapsulated system has shown to be effective in vitro.

Proposed Course:

They are presently planning to test their microcapsules in animals. They plan to study the physiology of the G.I. fluids and the behavior of these fluids with their microcapsules.

Contractor: Battelle Memorial Institute

Amount: $30,040

Title: Cannulae and Nonthrombogenic Materials

Objective:

This is a new contract for the development of methods of modification of cannula surfaces with heparin and bactericides or bacteriostats. Heparinization will utilize an organic solvent containing an oil-soluble quartenary ammonium salt tridodecylmethylammonium chloride (TDMAC), followed by exposure to sodium heparin. In vitro tests for an anticoagulant effect include rotating recalcified ACD blood through test tubing, checks for hemolysis, Stypven times, and tests for adenine nucleotide. Various solvents and extraction methods will be compared. Substituted trialkoxysilanes, using phenolic, mercuric, and quartenary ammonium types will be compared for use as an antibiotic agent. In vitro testing is performed using inoculated agar into which are pressed strips of test material.

Major Findings:

Greatly improved surface smoothness has been achieved by using the performed complex (heparin - TDMAC) on silicone rubber. They demonstrated that when blood was passed over a surface a small amount of heparin was eluted. However, the elution was not continuous, but quickly subsided. There was no substantial difference between the untreated silicone rubber versus the heparinized silicone rubber in regard to blood surface interactions. The results of the tests from bactericidal impregnation of surfaces was completely inconclusive.

Proposed Course:

The contract has been terminated.

Contractor: Beth Israel Hospital

Amount: $26,445

Title: The Role of Guanidine Compounds in the Pathogenesis of the Uremic Syndrome

Objectives:

The investigator will quantitatively determine the levels of guanidine and the disubstituted guandidines in body fluids in patients with chronic renal disease.

Contractor: Peter Bent Brigham Hospital

Amount: $22,500

Title: Changes in Intellectual Ability and Performance Associated with Uremia and its Modification

Objective:
This is a contract to study the effects of uremia on cognitive, perceptual and memory functions, and modification by dialysis and transplantation. The investigator will administer and study the Continuous Performance Test and the Flicker Fusion Test, and other quantitative repeatable tests found suitable for evaluation of sustained attention, perception, motor coordination, response readiness and memory.

Major Findings:
Uremic patients perform poorly on the CPT. When dialyzed, they show improvement but do not return to normal, as do successfully renal transplanted patients.

Proposed Course:
To continue development of the CPT as a measure of efficacy of dialysis and a potential human behavioral bioassay.

Contractor: Peter Bent Brigham Hospital

Amount: $10,000

Title: Studies of Toxic Factors in the Plasma of Uremic Patients Utilizing a Tissue Culture System

Objectives:

The contractor has initiated studies to best define those factors which depress cellular proliferation in uremia with the use of a tissue culture system.

Major Finding:

Preliminary work has suggested that human myeloblasts may be a useful cell line to study in an attempt to use tissue culture as an assay method. However, recent work has been plagued by the system's lack of specificity and selectivity.

Proposed Course:

This contract will terminate in June 1971.

Contractor: The Bronx-Lebanon Hospital Center

Amount: $56,400

Title: The Origin and Effect of Guanidinosuccinic Acidemia in Uremia

Objectives:

The contractor will investigate the quantitative interrelationships of guanidino and urea precursors in blood and urine samples of uremic patients who have had their diets altered with specific amino acids and guanidinium precursors.

Major Findings:

The investigator has shown that guanidinosuccinic acid (GSA) is elevated in uremia, removed by dialysis and increased in normals who are fed a high protein diet. Work has also demonstrated that GSA induces a reversible thrombocytopathy which is correlated with changes in electron micrographs. He has recently noted that GSA levels are increased in animals when various urea precursors are fed to them but decreased when methionine is given. These findings have been reproduced in humans.

Proposed Course:

Studies will continue on the unique role of methionine as a possible substance that can reduce GSA production and thus reduce the frequency of dialysis. They also plan to assess the role of GSA in ATP-ase inhibition which may be responsible for some of the hematological abnormalities seen in uremia.

Contractor: The Brooklyn-Cumberland Medical Center

Amount: $22,000

Title: The Effect of Peritoneal Dialysis and Hemodialysis on the Level of Water Soluble and Fat Soluble Vitamins in the Blood of Uremic Patients.

Major Objectives:

To study the effect of hemodialysis and peritoneal dialysis on the level of water soluble vitamins in the blood of uremic patients was to ascertain the specific vitamins removed by dialysis, to measure the amount of depletion of body stores, and to assess the significance of the losses. In addition, they wish to make specific suggestions regarding vitamin supplementation required in these patients.

Major Finding:

Patients who did not receive vitamin C supplements demonstrated a consistent decrease in plasma ascorbic acid during each dialysis with a mean decline of 40± from the predialysis value. During the course of the study two patients demonstrated skin changes consistent with those that occur in ascorbic acid deficiency.

Proposed Course:

To continue studying vitamin depletion during dialysis. The main emphasis will be on the following vitamins: thiamin, riboflavin, folic acid, B12, E, A.

Contractor: Brooklyn Veterans Administration Hospital

Amount: $9,500

Title: Investigative Ellipsometry and Related Studies

Objectives:

Mechanism of action of heparin-bonded nonthrombogenic membranes and their component materials, under study or produced by contractors of the NIAMD Artificial Kidney - Chronic Uremia Program, are to be investigated in Dr. Vroman's laboratory by ellipsometry and related surface chemistry techniques.

Major Finding:

Among heparinized polymers (obtained from Batelle Memorial Institute) GMAC adsorbs larhe amounts of plasma protein, especially fibrinogen and globulins as well as platelets and red cells; heparin displaces the protein. TDMAC however, adsorbs less protein and fewer platelets, and is less affected by heparin. Collagen films adsorb moderate amounts of fibrinogen and also some proteins.

Proposed Course:

The major emphasis will be on studying collagen, ultrathin cellulose and polypeptide membranes by means of the ellipsometry and related surface chemistry technique.

Contractor: University of California, San Francisco Medical Center

Amount: $25,900

Title: Clinical Evaluation of the Western Gear High Performance Multiple Point Support Dialyzer

Objective:
This is a contract to evaluate the Western Gear High Performance Multiple Point Support Dialyzer (developed under the Artificial Kidney Program of N.I.A.M.D.) to determine the clinical and performance characteristics, to investigate the feasibility of its use in one-shift dialyzers, and to study the relationship between dialyzer creatinine clearance and the hours of dialysis required per week for adequate chronic hemodialysis therapy.

Major Findings:
Preliminary findings indicate that the performance characteristics vary little from device to device, with mass transfer capability between that of a Kiil and Coil.

Proposed Course:
To investigate the feasibility of its use in one-shift dialysis.

Contractor: University of California - Wadsworth V. A. Hospital

Amount: $88,000.00

Title: Evaluation of Low Protein Diet in Dialysis

Objectives:

The contractor seeks to examine the effect of low protein diets in uremia in both dialyzed and nondialyzed patients, and to evaluate the optimal protein intake of patients on hemodialysis. He is also studying the biochemical phenomenon of protein metabolism in uremia.

Major Finding:

Studies were continued on low protein diets; work was done on the combination of low protein diets with infrequent dialysis. Patients received dialysis only once per eleven days and did well when utilizing a 26 gram protein diet obtained by the electrodialysis of whey.

Proposed Work:

The group will continue its study of the combination of diet and dialysis and will also study amino acid requirements and metabolism in the chronically dialyzed patient. They plan a detailed study of tyrosine metabolism in uremia.

Contractor: Case Western University

Amount: $48,900

Title: Application of Laser Doppler Technique to Artificial Kidney Design Mass Transfer Near Membranes and Kenetics of Blood Coagulation .

Objectives:

The objective of this contract is to study the application of the Laser Doppler flowmeter as it relates to two practical problems of artificial kidney design: (1) the study of mass transfer to boundary layers adjacent to membranes, and (2) the study of kenetics of blood coagulation. This is a new contract and has just been initiated.

Contractor: Case Western Reserve University

Amount: $85,198.00

Title: Removal of Waste Metabolites from Dialyzing Fluid by Microencapsulated Reactants

Objectives:
To develop a new technique for removing waste metabolites from dialysing fluid by encapsulating specific reactants inside hollow polymeric spheres.

Major Findings:
The investigators have successfully developed techniques for encapsulating solids and liquids. Studies of Giordano oxystarch confirm its previously reported high affinity for urea and/or ammonia.

Proposed Course:
Continued *in vitro* studies of encapsulation techniques and characterization of encapsulated materials.

Contractor: Case Western Reserve University

Amount: $61,449.00

Title: Research Leading to an Improved Artificial Kidney

Objective:
To develop improved cellulose acetate dialysis membranes.

Major Findings:
Techniques were developed for reproducibly making modified cellulose acetate membranes having water permeabilities 20-fold higher than that of Cuprophane, adequate albumin retention, and dialysis coefficients 1.5-fold higher than those of Cuprophane. Reinforcement with inexpensive multifilament nylon has given greatly increased strength and tear resistance.

Proposed Course:
Specific attention will be paid to finding better swelling promoters for cellulose acetate. In addition, control will be obtained over gellation by controlling the rate of solvent and swelling promoter removal during the immersion step. Variation of hydroxyl/acetyl ratio will be studied to give higher solute/water permeability ratios. Blood compatibility will be characterized, since preliminary measurements are promising.

Contractor: Cedars-Sinai Medical Center

Amount: $134,650

Title: Gastrointestinal Use of Sorbents

Objectives:

Determine the feasibility of using adsorbents in the gastrointestinal tract to treat patients suffering from chronic uremia. The adsorbents will be used in an attempt to decrease the urea load on the kidneys by enzymatic breakdown of the urea and adsorption of the products of this reaction within the gastrointestinal tract. An added dividend of this work may be its application for therapy of excessive serum and gut ammonia levels as found in hepatic failure cases.

Major Finding:

Initial studies of giving uremic dogs zirconium phosphate and activated carbon were not successful in lowering their BUN.

Proposed Work:

Studies on the physiology of the GI fluids in uremic and normal subjects will be undertaken. The behavior of the sorbent with the GI fluids will also be studied.

Contractor: Cedars-Sinai Medical Center

Amount: $200,000

Title: Osteodystrophy and Divalent Ions in Kidney Failure

Objectives:
Perform a systematic and comprehensive study to evaluate the mechanism leading to deranged divalent ion metabolism in renal failure.

Major Findings:
Evaluations of mechanisms involved in the development of abnormalities of divalent ion metabolism in 1) stable chronic renal failure patients; 2) patients undergoing chronic hemodialysis; 3) patients from post-renal homotransplantation; and 4) patients with early minimal degrees of renal failure in approximately twenty patients per group. Investigation into the role of Vitamin D resistance and altered parathormone activity in producing the skeletal and soft tissue pathology indicates that a progressive Vitamin D resistance occurs and skeletal unresponsiveness to parathormone in conjunction either initiates or aggravates the osseous and soft tissue abnormalities.

In addition the investigators have found higher levels of skin calcium in patients dialyzed with higher dialysate calcium concentrations, although phosphate intake was uncontrolled.

Proposed Course:
Continue investigations into the mechanisms of divalent ion metabolism in uremic osteodystrophy with application of findings toward rational modes of therapy for this disorder.

Contractor: Cleveland Clinic

Amount: $55,000

Title: Development of an Envelope Kidney

Objectives:
1) To develop an envelope (presterilized) membrane for use in a Kiil; 2) To develop a coil envelope (low cost, convenient, presterilized); 3) To evaluate the feasibility of an intracorporeal artificial kidney.

Major Findings:
A Kiil-Envelope has been successfully developed and clinically tested and is ready for marketing to the public. The other two objectives are just beginning study.

Proposed Course:
Objectives (2) and (3).

Contractor: Columbia University

Amount: $169,700

Title: Identification of a Nonthrombogenic Environment

Objectives:
Identify those characteristics of surfaces and blood which make an atraumatic nonthrombogenic environment possible.

Major Findings:
Two intravascular test devices have been designed to permit in vivo testing of artificial materials under controlled blood velocities. Particular parts of the device are isolated from contact with possibly damaged intima and consequent release of tissue thromboplastin. The role of various surfaces, turbulent flow, platelets, coagulation factors, temperature, and blood velocity on the initiation of clotting are being investigated. Various histologic chemical and physical manifestions of clotting are being measured. The effects of aspirin and methylene blue on clotting are also being studied.

Proposed Course:
Continue this multifacet bioengineering approach in an attempt to better define an artificial nonthrombogenic system.

Contractor: Cornell University Medical College

Amount: $91,650

Title: Biologically Derived Collagen Membranes and Surfaces: Their Application to Problems of Hemodialysis and Chronic Uremia

Objectives:

Investigate the properties of dialysis membranes and A-V shunts constructed from solubilized collagen.

Major Finding:

Collagen membranes have been fabricated and tested on a limited scale. Dialysance value of urea, utilizing both paths of collagen coils, at blood flows of 200 ml/min is approximately 150 ml/min. Slight increases in permeability have been accomplished by making the membranes thinner. However, the thinner membranes have been plagued by pinholes.

Proposed Course:

Major emphasis will be directed towards testing the collagen membranes.

Contractor: The Dow Chemical Company

Amount: $56,500

Title: Research and Development of Cannulas and Nonthrombogenic Materials

Objectives:
Develop, fabricate, and evaluate a useful cannula for subcutaneous insertion between the arterial and venous ends of vessels. Continue investigation of dacron velour with appropriate diameter and thicknesses for inhibition of infections. Investigate coating of silastic cannulas with nonthrombogenic materials.

Major Findings:
1. It was found that the end to side anastomosis is apparently not feasible in small peripheral arteries with this investigating group.

2. Prototypes of the proposed cannulae are being fabricated and will undergo modification and in vivo testing in laboratory animals in the ensuing months.

Proposed Course:
Continue development of cannula design and test with nonthrombogenic material linings.

Contractor: The Dow Chemical Company

Amount: $183,000

Title: Research on Hollow Fiber Dialyzer Unit

Objectives:
To develop a hollow fiber dialyzer as an efficient low-cost artificial kidney.

Major Findings:
The hollow fiber hemodialyzer has become a commercial device over this past year and is currently widely available to the consuming public. Continuing studies on thrombosis indicate that endotoxins involved in the manufacture of the hollow fiber kidney play a major role in ensuing thrombosis of the kidney. Steps are being taken to eliminate any non-sterile manufacturing process of the kidney.

Proposed Course:
Continue development of hollow fiber dialyzers with emphasis on more permeable membranes and more advantageous control of the hydraulic permeability. There will be continued development of a small highly portable artificial kidney which may be interdigitated with a regenerative sort of system for the reuse of dialysate. There will be continuing investigation into the problems and potential solutions to the problems of thrombus formation in the artificial kidney.

Contractor: Albert Einstein College of Medicine

Amount: $50,000

Title: The Structure and Function of the Hepatic Endoplasmic Reticulum in Chronic Renal Failure

Objectives:

Objective of this new contract is to investigate the role that chronic uremia has in relation to the function of the hepatic endoplasmic reticulum. It is further hoped that through elucidation of the role of the endoplasmic reticulum an understanding may be made of the mechanisms of drug metabolism in chronic uremia.

Contractor: Gulf South Research Institute

Amount: $93,000

Title: A Study of Synthetic Polypeptides as Possible Hemodialysis Membranes

Objectives:

To form membranes of diverse amino acid polymers and conduct studies directed at an evaluation of their in vitro mass transfer, physical, and clot-promoting characteristics, and do in vivo clinical evaluation studies.

Major Findings:

Polypeptide membranes have been prepared during this year from copolymers of leucine and methionine poly-methyl-D-glutamate.

A number of membranes have been prepared and they have been characterized by their mass transfer properties concurrently with their blood compatibility.

Mass transfer measurements of the above membranes were made using NaCl, creatinine and bacitracin. Polypeptide membranes were formulated which provide dialysis rates at least as good as Cuprophane on the usual smaller molecular weight compounds, and, in the particular case of bacitracin, permeability data was obtained indicating this membrane to be better than Cuprophane.

Considerable effort was expended in developing membrane fabrication techniques so that sufficient material would be available for the in vivo clinical evaluation testing.

Proposed Course:

The primary objective of the project is the development of an improved membrane material suitable for hemodialysis. Scaled-up procedures for membrane fabrication are being developed for membranes from poly-methyl-D-glutamate and leucine-methionine polymers. Further in vitro characterization of these membranes will be done. In vitro thrombogenicity testing of these materials will continue.

In vivo clinical evaluation studies are now being organized.

Contractor: Envirogenics Company

Amount: $19,800

Title: Development of an Inexpensive Manufacturing Method for the Kiil-Type Artificial Kidney

Objectives:

To develop a low-cost manufacturing process for the Kiil-type artificial kidney.

Major Findings:

The production of Kiil-type boards, using advanced mold making and molding techniques, to both decrease the production costs and improve reproducibility has been accomplished. These techniques comprise the combination of precision grinding methods and electroforming for mold making processes and precision compression molding to provide finished molded parts with accurately reproduced detail dimensions, surface finish and flatness.

A number of hemodialyzer units have been fabricated and tested in vitro and are about to undergo in vivo tests. It has been found that Kiil type kidney boards can be molded readily and, therefore, theoretically should result in a substantial cost reduction. The technological system used in this study will result in molded boards which are about 25% lighter than machines ones, thus facilitating handling. Full scale production tooling utilizing these technological techniques are in the final stage of completion and may be used to fabricate practical Kiil type boards with optimum efficiency. Several advantages accrue from this format: low cost, 25% reduction in weight, and a very dimensionally stable blood path.

Proposed Course:

Clinical Evaluation of Molded Kiil Kidney Boards - Tests are to be made to compare the molded and the classical Kiil board as they relate to performance and ease of handling.

Contractor: Franklin Institute

Amount: $20,700

Title: Cannulas and Nonthrombogenic Materials

Objectives:
Develop a system for the permanent implantation of cannulae for chronic hemodialysis.

Major Findings:
Cannula tips have been fabricated of a variety of synthetic materials in an attempt to provide adequate device-vascular and device-dermis matching. The percutaneous device was an interesting composite in an attempt to provide proper modulus matching and compliance between the device and the skin. Geometrical factors of cannulae appear not to be of major significance in the longevity of the cannulae compared to techniques of implantation. In vivo studies in lab animals were carried out.

Proposed Course:
No significant advances were made during the award period and the contract has been terminated.

Contractor: Georgetown University

Amount: $53,400

Title: New Approaches to Design of Chronic Dialysis Cannulae for the Prevention of Infection

Objectives:

1. Develop a method of fixation of an AV cannula to minimized turbulence related thrombosis in cannula dislodgement due to vessel nephrosis.

2. Develop an antithrombogenic material for use in the AV cannula.

3. Prevent cannula sinus tract formation by means of developing a percutaneous seal for the skin prosthetic junction.

Major Findings:

Arterio-venous shunts coated with hepacone have proven to be thrombo resistant in dogs and a unique vascular shunt anastomosis method has reduced junctional clotting in animal experiments. A novel percutaneous button seal has been developed and tested in animals and appears to be a promising approach to prevent infection of the sinus tract formation. The shunt survival with this group seemsto be as great as with any group investigating cannula survival at the present time.

Proposed Course:

Continue development of a tissue compatible nonthrombogenic AV shunt. Continued clinical trials will be carried out.

Contractor: I. I. T. Research Institute

Amount: $48,7000

Title: Development of an *in vitro* assay for toxic components in uremic serum

Objective:
This is a contract to determine the average rate of C-14 labelled AIB transport by normal rabbit renal tubules in normal and uremic human sera to determine if uremic toxins exert a significant inhibitory effect.

Major Findings:
A small difference of AIB uptake in tubules suspended in normal vs. uremic sera was identified. This contract is being terminated.

Contractor: I. I. T. Research Institute

Amount: $55,500

Title: Fractionation and Identification of Metabolites Elevated in Uremia

Objective:
The investigators under this contract undertook to isolate and identify UV-absorbing substances that are present in higher concentrations in the urine and dialysate of some uremic patients than in urine of normal controls.

Major Findings:
Uremic dialysates or urine were chromatographed on columns of Bio-gel P-2. The materials of primary interest eluted just prior to the uric acid peak and just after, and exhibited higher absorption at 260 nm than at 280 nm. A comparison of patterns from normal and uremic urine suggested these two fractions as being characteristic of the uremic state. Preliminary tests to characterize these fractions included absorption spectra at different pH values for acid hydrolyzed and unhydrolyzed samples. The fractions were also chromatographed in three different solvent systems. The fraction eluted prior to uric acid, upon acid hydrolysis, resulted in an ultraviolet-absorbing peak corresponding to the peak eluted after uric acid, and an additional component that is ninhydrin positive.

Contractor: Thomas Jefferson University - Jefferson Medical College

Amount: $18,349

Title: A Clinical Study of Automated Chronic Peritoneal Dialysis

Objectives:

The new contract will perform a clinical trial of the investigators' newly developed automatic chronic peritoneal dialysis instrument on patients who were refused hemodialysis.

Contractor: Thomas Jefferson University - Jefferson Medical College

Amount: $23,300.00

Title: The Isolation, Identification and Quantitative Evaluation of Lipochromes and Urochromes in Normal and Uremic Patients

Objectives:
The objective of this contract is to investigate, characterize and quantitate plasma lipochrome and urinary urochrome levels in normal patients and uremics and to develop methods for their analysis.

Major Findings:
The contractor has devoted most of his time to perfecting and standardizing techniques for the analysis of lipochromes. Plasma levels have been measured in uremic and normal patients. The lipochromes were recently identified as unsaturated long chain fatty acids which are completely peroxidized.

Preliminary tests with a vitamin E deficient diet (vitamin E is the major antiperoxidant in the body) have demonstrated anemia and neuropathy in dogs.

Proposed Course:
The investigator believed the presence of lipochromes is a result of faulty antiperoxidation. He intended to measure vitamin E levels in uremics to determine if there is a deficiency of this compound, and to reproduce uremic symptoms using vitamin E deficient animals to determine the role of this virtually unstudied compound in uremia.

However, during the course of this work the principle investigator died and the contract was subsequently terminated.

Contractor: Johns Hopkins University School of Medicine

Amount: $78,500

Title: Metabolic Studies in Uremia

Objectives:

This is a new contract to investigate the physiological characteristics of the anephric state, paying particular attention to hormone functioning. Specifically, adrenal function will be evaluated by a study of cortisone and aldosterone metabolism. Attempts will also be made to elucidate the mechanism of hypoglycemia in uremia and to distinguish it from the hypoglycemia of diabetes mellitus.

Major Findings:

Anephric patients showed no response to upright posture and exhibited higher concentrations of plasma aldosterone on the third and fourth day post-dialysis. However, it is thought that aldosterone secretion is responsive to other stimuli. The results suggest that the primary regulator of aldosterone secretion in the absence of the kidneys is potassium.

Proposed Course:

Work will continue on studies of cortisol clearance, cortisol production rates and variations in free and bound cortisol in anephric patients and patients with advanced renal insufficiency who have not been subjected to bilateral nephrectomy.

Contractor: Massachusetts Health Research Institute

Amount: $39,000

Title: Low Protein Diet in Uremia

Objectives:

This is a new contract to determine if a high biological value protein diet based on whey can be used to decrease the frequency of need for hemodialysis in chronic renal failure; to devise a diet that is appetizing as well; to determine if certain amino acids are more toxic than others. After obtaining baseline data on about 20 patients, they will be placed on a whey diet so that dialysis will be required only every seven to fourteen days. In addition to following routine blood chemistries and clinical symptoms, serum amino acids and nitrogen balance will be measured. Food substitutes and amino acid additions will be made for evaluation of individual effects.

Major Findings:

The group claims to have lengthened interdialysis intervals by using a high biological value protein diet composed primarily of electrodialyzed whey (EDW). However, their protocol for selecting patients was inadequate to give conclusive results. The rat studies have not demonstrated anything to date.

Proposed Course:

The contract has been terminated.

Contractor: Arthur D. Little Incorporated

Amount: $38,200

Title: Elucidation of Toxic Nature of Uremia by Application of the Spin Filter Culture System

Objectives:

To elucidate the toxic nature of uremia by application of the Spin Filter Culture System. By means of this system, mammalian cells can be propagated in suspension and the uremic toxins present in dialysate, may be chronically administered to these cells.

Major Finding:

Normal dialysate and dialysate with an increased concentration of urea and creatine were found to have no adverse effect on the Spin Filter culture system.

Proposed Course:

To test dialysate from uremic patients in the Spin Filter Culture System.

Contractor: Massachusetts Institute of Technology

Amount: $87,227

Title: Research and Development Directed Towards the Design of an Artificial Kidney

Objectives:
To develop non-thrombogenic surfaces for a pilot model of an artificial kidney.

Major Findings:
Studies of nonthrombogenicity of formaldehyde-glutaraldehyde-hydrogels covalently bonded with heparin indicate that it is a highly unusual material of low thrombogenicity.

Proposed Course:
Hydrogels of this type will be made into membranes, cannulae and catheters and characterized for potential clinical usage.

Contractor: Mayo Foundation

Amount: $150,000

Title: Value of Maintaining Parathyroid Hormone Suppressive Calcemia in Prevention of Bone Disease and Abnormalities in Calcium Hemeostasis in Patients on Long-Term Hemodialysis

Objectives:

This project is directed toward assessing the value of maintaining physiologic plasma concentrations of both calcium and phosphate in suppressing parathyroid hormone secretion and preventing metabolic bone disease and abnormalities of calcium homeostasis in patients maintained on long-term hemodialysis. Parathormone assays in relationship to varying levels of dialysate calcium, dietary calcium, and possible vitamin D supplementation will be studied. Parathormone assays in relationship to phosphate which had been lowered by oral administration of aluminum carbonate and hydroxide will also be studied. Bone biopsies and bone density studies will be performed.

Major Findings:

Thus far, the dialysate Ca level has been identified as a critical factor in the pathogenesis of renal osteodystrophy. In addition, it has been found that maintenance of serum phosphate near normal levels with phosphate-binding gels is crucial. Serum PTH levels respond to changes in these critical factors.

Proposed Course:

Evaluation of dialysate Ca concentration of 8 mg %, its effect on PTH, and follow-up of patients receiving renal transplantation.

Contractor: Milton Roy Company

Amount: $28,000

Title: A Program for Clinically Evaluating an Advanced Single-Patient Hemodialysis System

Objectives:

To develop an automated, "Fail-Safe," dialysate delivery system.

Major Findings:

Following the development of a satisfactory dialysate delivery system prototype, ten such models were fabricated and underwent rigorous clinical evaluations in five dialysis centers, engaged both in home and hospital dialysis programs. Parameters such as size, safety, operational simplicity, reliability, serviceability, cost, and patient acceptance were monitored.

The consensus of opinion for this clinical evaluation testing program was that the servo controlled proportioning system performed very well, but that the automatic cycle sequence programming was of marginal value. The patients and technicians found it difficult to understand, adjust and maintain the rather complex electronics associated with the servo controlled proportioning and automated programming.

This contract has been terminated.

Contractor: Minneapolis Research Foundation

Amount: $56,200

Title: Investigations of Nutritional Requirements of Chronic Renal Failure

Objectives:
This is a contract designed to delineate nutritional requirements of patients with chronic renal failure treated conservatively and by long-term hemodialysis including dietary protein requirement to maintain optimal body composition and body weight as well as specific amino acid requirements.

Major Findings:
This contract has recently been finally initiated and at the present time the investigator has finish equipping the lab necessary for the study of the objectives. The clinical investigation is beginning at the present time.

Contractor: Montreal General Hospital

Amount: $57,600

Title: Determination of Nutritional Requirements and the Study of Body Composition in Patients on Chronic Hemodialysis

Objectives:

Dr. Kaye's objectives are to determine the nutritional requirements and to study the body composition of patients on chronic hemodialysis.

Major Findings:

The investigator has demonstrated that renal osteodystrophy may respond well to dihydrotachysterol (DHT) and he has successfully reversed progressive bone disease in dialyzed patients studied thus far. He has also studied fluoride levels in patients undergoing dialysis in both fluoridated and non-fluoridated areas and has found no difference in the degree and type of bone disease in these two populations.

In addition he has undertaken preliminary animal studies using the uremic rat.

Proposed Course:

Extensive animal model studies to systematically determine the causative factors of renal osteodystrophy in well-controlled studies.

Contractor: University of Naples

Amount: $24,500

Title. Studies on Oxystarch in the Treatment of Uremia

Objectives:
To develop a chemical compound which can be administered per os to sequester urea and possibly other uremic toxicants in the gastrointestinal tract, and to develop with the aid of such a compound a suitable adjunctive treatment for patients in chronic renal failure. Also, to explore the feasibility of using oxystarch as a sorbent of uremic wastes in artificial kidney dialysate solutions.

Major Findings:
A chemically well defined polyaldehyde has been obtained from controlled periodate oxidation of starch. The material does not contain iodine residues and has been proven nontoxic in long-term mouse feeding experiments. The material is stable and non-dialyzable. One hundred grams of "oxystarch" bind 33 grams of urea. When given by mouth to uremic patients in divided doses, 20 grams of oxystarch per day result in a daily reduction of 20 milligram percent of blood urea nitrogen. Fecal nitrogen content increases correspondingly and the increased fecal nitrogen can be ascribed to large quantities of urea irreversibly trapped by oxystarch during passage of the gut. Oxystarch is not hydrolyzed in the intestinal tract, nor absorbed. Twenty grams of oxystarch when given daily in three to four divided doses to uremic patients maintained on the Giordano-Giovannetti diet caused a progressive daily lowering of blood urea nitrogen of 13 milligram percent (range 8 to 22); the corresponding increase in nitrogen content of the feces had a net mean value of 1,450 milligrams per day (range 730 to 8,050). When oxystarch was given to a patient on regular 3-times-weekly hemodialysis, the interdialytic interval could be prolonged up to 7 days without untoward effects. When oxystarch was given to a patient maintained with weekly peritoneal dialysis it was possible to space these treatments 25 days apart.

Dr. Giordano's laboratory data on urea binding with oxystarch at pH 1 to 1.2 and pH 7.4 have been corroborated by Dr. Robert Sparks at Case Western University in in vitro experiments.

Proposed Course:
The investigator will test the use of OXS in controlled larger clinical trials for prolonged periods of time in accordance with carefully outlined plans. Also, (1) long-term animal toxicity studies will be concluded, (2) oxystarch, charcoal and celite will be tried as regenerating agents in the circuit of a recirculating artificial kidney operating with a very small dialysate bath volume, and (3) the contractor will prepare oxystarch in quantities large enough for clinical and laboratory experimentation in the United States as specified by the Artificial Kidney - Chronic Uremia Program which will distribute this material to American investigators for specific, sponsored studies.

Contractor: University of Naples

Amount: $24,651

Title: Studies on Amino Acids and Uremia

Objectives:
To investigate the possibility of extending the interval between dialyses in patients with chronic uremia through the use of the Giordano - Giovannetti diet suitably enriched with additional high quality protein to compensate for amino acid losses at the time of dialysis. To study in detail selected facets of amino acid metabolism in chronic uremia with emphasis on phenylalanine, tyrosine and histidine.

Major Findings:
Nitrogen losses during standard hemodialysis treatment were determined in a series of steady state dialysis patients and divided into waste nitrogen and amino acid nitrogen. It was found that an average of 25 grams of amino acid protein is lost during each dialysis treatment. Based on a nitrogen requirement in uremia of 0.3 grams of protein per kilogram of body weight and the above-mentioned amino acid loss during dialysis, the classical Giordano high-quality, low-protein diet was modified to permit maintenance of uremic patients through a combination of diet and infrequent hemodialysis. For a 70-kilogram patient the modified dietary regimen was one in which between 35 to 40 grams of high-quality protein were given each day (the precise number depending on the frequency schedule of dialyses). Calories were supplied at the rate of 35 per kilogram of body weight.

It was found that this combination regimen was suitable for 30% of the dialysis patients in the University of Naples center. At this point the investigator has patients who require dialysis only once a week, others who require it every five days, and others who require it twice a week. Thus far there has been no evidence of untoward side effects (especially peripheral neuropathy) among the patients on a reduced dialysis schedule and the investigator feels that in selected patients a suitably modified, high quality, limited protein diet can be instrumental in reducing the number of dialyses required for treatment of patients in chronic uremia.

Exploratory studies on histidine metabolism in uremia have shown evidence that histidine supplementation might be of practical value in uremic patients. In previous experiments Dr. Giordano had found that this semi-essential amino acid [essential for growing children, but not for grown adults] is present in uremic plasma in reduced levels. In a series of experiments involving the uptake of deuterium-labeled leucine by reticulocytes from normal controls and uremic patients on different well-defined diets, it was possible to show that dietary supplementation with histidine could double the amount of leucine incorporation into these cells in patients on low protein diets. This indicates that histidine supplementation might be beneficial in counteracting uremic anemia in patients on a low protein intake. In concurrent nitrogen balance studies, histidine supplementation also coincided with nitrogen balance improvement in these patients.

Proposed Course:

The above-mentioned combination regimen of infrequent dialyses and the Giordano - Giovannetti diet will be continued so that experience might be established concerning how effective such maintenance treatment can be, how safe it is, and for over what periods of time. Also, the preliminary results obtained with histidine supplementation will be followed into the clinical, applied sphere.

Contractor: National Institute for Scientific Research

Amount: $68,400

Title: Development of Modified Polycarbonate Membranes for Hemodialysis

Objectives:

To develop high strength polycarbonate membranes for hemodialysis combining the excellent mechanical properties of commercial aromatic polycarbonates (e.g., Lexan) with the transport properties of more hydrophilic gel membranes.

Major Findings:

In a preliminary feasibility study, a series of eight Bisphenol-A-copolyether-carbonates were prepared containing either 25% or 50% by weight of the hydrophilic segment polyoxyethylene glycols (PEG 600 or PEG 6000). Thus the effect of block molecular weight (m.w.) was evaluated. In addition all aromatic-linked copolymers were prepared by phosgenating prepolymers of PEG 600 (and PEG 6000) with Bisphenol A. This permitted a comparison of hydrolytic stability between all aromatic ester groups and random aromatic-aliphatic types.

Property trends measured for both series of polymers compared with pure Bisphenol-A-polycarbonate were clear. Water sorption increased with increasing percent of hydrophilic component, the higher m.w. block being more efficient. No differences were observed between all-aromatic and random aromatic aliphatic types. Mechanical strength of the 25% copolymers was equivalent or slightly less than that of pure Bisphenol-A-polycarbonate, while the 50% copolymer was poorer. Cast films of the 25% copolymers showed ideal behavior while the 50% polymer films were overswelled in water.

The dialysis data for a complete series confirmed the predictions. Commercial Lexan polycarbonate had no water transport, and infinite resistance to sodium chloride. Copolymer films containing 25 and 50% of PEG 6000 had ultrafiltration rates 25 times that of Cupraphane and salt permeabilities within an order of magnitude of Cupraphane. Little difference was noted between the two films.

Proposed Course:

The continuing effort is to focus on further refining the relationships studied to date, namely (1) size and number of hydrophilic blocks required for optimum transport properties (2) molecular weight and its relationship to film strength. In addition, several new aromatic diols will be studied since these provide another route to high strength polymers of enhanced water sorption. A third approach to modifying film properties will be through formulation of the new polymers into swelled membranes. Some 25-40 copolymers will be evaluated in dialysis equipment, as well as by classical polymer characterization procedures. The response of these new species to Heparin will also be studied.

Contract: New York Medical College

Amount: $160,561

Title: The Use of a Special Protein Restricted Diet in Uremia and the Mechanism of Its Effectiveness

Objectives:

The original objective of this project was evaluation of a special protein restricted diet for the alleviation of the subjective and objective signs and symptoms of uremia and an attempt to use this diet to elucidate the toxic factors in uremia. This objective has been expanded to include a search for the toxic factor(s) responsible for the transketolase inhibitor in uremic serum.

Major Findings:

In the first four years of this contract the New York Medical College group have treated 70 patients with a modified Giovannetti-Giordano diet. In patients with GFRs of greater than 4ml/min, dietary therapy was usually adequate in reducing gastrointestinal symptoms and increasing the patients' comfort although it in no way slowed the progress of the underlying renal disease or its complications. Studies were also undertaken to prolong the interval between dialyses in patients with GFRs of less than 4 ml/min. Diet-dialysis therapy was always associated with the eventual development or worsening of motor neuropathy which required intensive dialysis for reversal. Pre- and post-dialysis serum amino acid levels have been determined in 17 patients.

Because neurological signs and symptoms play such an important role in the uremic syndrome, and because some of the neurological manifestations in the patients resembled those noted in other conditions, the investigators recently began a search for identifiable metabolic correlates which might in some way explain the neuropathy. The investigators discovered that transketolase activity was found to be reduced by an inhibitory factor acting directly on transketolase which is found in the blood and dialysates of uremic patients. Transketolase was determined in 15 chronic uremic patients before and after four to six hours of hemodialysis and in four patients with advanced renal disease before and after institution of G-G diet therapy. In all except two patients on hemodialysis there was a significant increase in transketolase activity after dialysis. In patients on the G-G diet, transketolase values were initially low and remained low even after considerable time on the diet (36 weeks in the case of one patient). The responsible factor(s) was concentrated and separated into various molecular weight fractions. Only fraction I (mol wt less than 500) produced significant inhibition.

The results of these investigators indicate that a substance or substances inhibiting the activity of the enzyme transketolase, for

which thiamine pyrophosphate is the coenzyme, is found in the plasma of chronic uremia patients. This inhibitor is not significantly diminished by diet therapy, but can be removed by hemodialysis. The enzyme affected occupies a central position in the pentose phosphate pathway of non-oxidative glycolysis. The demonstration of an inhibitor of erythrocyte and brain transketolase in plasma from uremic patients suggests a possible mechanism for the development of uremic neuropathy, and its response to hemodialysis. The contractor feels that this enzyme model system may offer a technique for investigating the metabolic lesions of uremia and may serve as an objective measure of response to dialytic therapy.

Proposed Course:

The investigator will continue studies to identify the toxic materials and extend the clinical and biochemical evaluation of the transketolase inhibition.

Contractor: University of North Carolina

Amount: $47,200

Title: Antithrombogenic Surfaces: Platelet-Interface Reactions

Objectives:
Study the composition of the layer of plasmatic components adsorbed to materials exposed to blood, and to characterize this layer as to its effect on blood platelets.

Major Findings:
These investigators indicate that there are morphological differences between the adsorbed layers formed on certain polymeric and nonpolymeric test materials and that an adsorbed layer is universally present between adherent platelets and the surface of the test material. It appears that it is this adsorbed layer and not the surface of the test materials which determines thrombogenicity. The chemical nature of the test material may control the composition of the adsorbed layer and through this, its thrombogenicity, or it may merely control the rate of deposition of the adsorbed layer.

Proposed Course:
Continue characterization of the blood-polymer layer and its role in material thrombogenicity. He is also going to evaluate various polymers in an *in vivo* situation by placing vena cavae rings in Rhesus monkeys.

Contractor: North Star Research and Development Institute

Amount: $88,200

Title: Development of a New Concept in Membrane Structure for Application in Hemodialysis

Objectives:

The objective of the contractor has been to develop ultrathin (5,000-20,000 angstroms in thickness) membranes for hemodialysis.

Major Findings:

The most promising membrane discovered thus far is ultrathin cellulose. Ultrathin cellulose (when supported) had approximately twice the dialysis rates of cuprophane and about three times the ultrafiltration rate of cuprophane. The membrane did not pass albumin and had approximately equal dialysis permeabilities for bacitracin. In addition, the contractor has developed techniques that enable large amounts of the ultrathin polymer to be manufactured at minimal cost. This would enable the membrane to be useful commercially.

Proposed Course:

In vivo animal testing of the membrane and support composite in preparation for clinical testing.

Contractor: University of Pennsylvania

Amount: $33,697

Title: Development and Testing of a Clinical Model of a Disc Cone Dialyzer

Objectives:
Develop a clinical model of a radial flow dialyzer utilizing a cone membrane support in order to provide efficient membrane scrubbing, uniform distribution of blood and dialysate, optimal combination of blood flow, blood volume, pressure drop and blood path thickness.

Major Findings:
A model has been satisfactorily constructed and tested in vitro and in vivo. With a membrane surface area of 0.8 m^2, mass transfer data were near that of a Kiil. The objectives fulfilled, this contract is being terminated.

Contractor: University of Pennsylvania

Amount: $154,000

Title: Blood Purification by Ultrafiltration and Reconstitution

Objectives:

To study the factors involved and develop a system for cleansing the blood by ultrafiltration and replacing the ultrafiltrate with sterile reconstituting solution containing appropriate concentrations of normal body constituents removed in the ultrafiltrate.

Major Findings:

Work has been largely completed on the theoretical formulation of concentration polarization of ultrafiltered protein containing solutions. This phenomenon was previously identified as the single most important limitation encountered during the first year's study of the feasibility of diafiltration as blood cleansing technique. The concentration polarization model formulated has been tested by varying such parameters as temperature, shear rate, protein concentration and pressure. Ultrafiltration rate was found to rise with increases in working temperature and blood path shear rate, to fall with increasing protein concentration and remain unchanged when transmembrane pressure gradient is varied above a plateau figure of 15 p.s.i. This performance substantiated the validity of the concentration polarization model.

A hollow-fiber configuration has been utilized in animal studies which have demonstrated the feasibility of acute hemodiafiltration. Clearance of large molecules (MW 5000) was nearly 90%, and that of smaller molecules nearly 100%.

A reconstitution system and a flow-pressure monitoring system has been developed that is operationally successful.

Proposed Course:

Additional animal studies will be undertaken to prevent potential toxicity. A clinically practical reconstitution system will be explored, and clinical testing will evaluate the efficacy of the process.

Contractor: University of Pisa

Amount: $26,985

Title: Effect of the Amount and Quality of Dietary Proteins on the Uremic Syndrome; Effect of the Dietary Treatment on Albumin and Urea Metabolism in Chronic Uremia

Objectives:
To perform a well controlled in-house study of the effect of the amount and quality of dietary proteins on the uremic syndrome and to determine the effect of the dietary treatment on albumin and urea metabolism.

Major Findings:
Comparison, in the same patients, of the effect on the uremic syndrome of a diet containing 45 grams of unselected protein (one-half of animal origin, the other half biologically poorer cereal proteins) with a diet containing only 18 grams of protein of the highest biological value has shown the following: (1) In the former case, a deterioration of blood chemistries in all patients with greatly increased urea production, accompanied by a severe clinical deterioration. (2) A crossover to the latter, 18-gram selected protein diet resulted in a prompt improvement of blood chemistries and the clinical picture.

Sophisticated determination of albumin catabolism through a new triple tracer technique has shown that the intravascular albumin mass is normal in patients on the low, selected protein (Giordano-Giovannetti) diet (even in patients maintained on it up to five years). The extracellular albumin mass, total albumin mass and extracellular to intravascular ratio were reduced in both short-term and long-term patients. The total intravascular albumin mass was preserved within a normal or near normal range. Albumin metabolism was normal in controls and long- or short-term patients. The diet is not directly responsible for the observed albumin depletion since albumin synthesis continues at a normal rate in all cases. Apparently toxic factors of the uremic state itself are responsible.

Proposed Course:
Continue to evaluate albumin metabolism in patients who are uremic and on a low protein diet, investigate the urea metabolism in these patients, and study albumin metabolism and distribution in uremic patients who are on hemodialytic therapy.

Contractor: University of Pisa

Amount: $20,222

Title: Role of Guanidines and Related Compounds in Uremic Syndrome

Objectives:
To investigate the role of guanidines and related compounds in uremic syndrome, specifically in relation to the hemolytic and bleeding tendency, central and peripheral neuropathy, glucose metabolism, calcium metabolism, lipid metabolism, and immunologic response.

Major Findings:
Methylguanidine (MG) was retained in man in chronic uremia, and when administered to dogs for two to three weeks induces the appearance of symptoms resembling those of uremia: protein catabolism, anemia due to hemolysis and decreased red cell production, vomiting, diarrhea with ulcerations and bleeding in the stomach and duodenum, tremors, convulsions, peripheral neuropathy, alveolar hemorrhages and edema. Subsequent studies in dogs chronically intoxicated with MG showed decreased intestinal calcium absorption and mild skeletal intoxication. In such dogs it was shown that during MG intoxication triglyceride values increased significantly and those of cholesterol decreased to yield a blood lipid picture characteristic of uremia and that intoxicated dogs had a highly depressed immunologic response (reminiscent of the impaired immunologic capability in uremia). Other experiments were carried out whose results confirm that urea is a relatively nontoxic metabolite.

A new and very satisfactory procedure for the specific determination of MG in biological fluids has been developed and a paper describing it has been received. In experiments in normal human controls it was clearly shown that the urinary excretion of MG is much higher on a regular mixed diet than on the Giovannetti low protein diet. The renal output of MG in renal patients was followed under a variety of dietary conditions. These experiments showed a parallel lowering in plasma urea and methylguanidine when patients were on the G-G diet, as well as a decreased urinary MG output. This is consistent with the hypothesis that the improvement in the clinical condition which usually occurs in uremic patients on the G-G diet may be related to a decrease in plasma concentrations of MG. It was also shown that the amount of MG produced was higher if the dietary proteins were furnished by meat rather than by milk and eggs. Preliminary experiments have been started to test the possible adsorption of MG with the aid of charcoals and exchange resins from biological fluids in vitro.

Proposed Course:
The research work projected for the coming year consists of four parts: (1) combined intoxication with urea and methylguanidine in dogs; (2) additional attempts to pinpoint the toxic factors which are responsible for the disturbances in the carbohydrate metabolism of uremia; (3) confirmation, during longer experimentation, of some results of the previous dog work, followed by (4) confirmation, where possible, of previous animal experimentation concerning MG, in man, with emphasis on possible improvements in the

conservative treatment of uremia. One aspect of this work concerns the selection of dietary nitrogen sources which will not contribute MG or MG precursors to the patient.

Contractor: University of Portland

Amount: $25,425

Title: An AK - Utilizing a New Ultrafiltration Technique

Objectives:

This is a new contract to study the use of desiccants on the outside of semi-permeable membranes for the purpose of removing water and associated small ions and molecules from flowing blood. Consideration will be given to techniques such as hydrolysis of urea by urease and subsequent trapping of ammonia to accelerate urea transport. The objective is a device which is able to supplement presently available artificial kidneys by "removing some water and small molecules as desired."

Major Findings:

He was able to remove approximately 1400 grams of water in 6-8 hours using his UF method and polythylene glycol as the desiccant. However, the PEG diffused through the membrane of the unit. This presents a potential toxicity problem. This method is thought not to represent any major improvement in the area of ultrafiltration in that a standard hemodialyzer can remove an equal or greater quantity of ultrafiltrate.

Proposed Work:

None. The renewal proposal has not been approved.

Contractor: Research Triangle Institute

Amount: $90,000

Title: Improved Nonthrombogenic Materials

Objectives:

This is a new contract to prepare a number of surfaces by grafting hydrophilic polymer chains to a solid non-polymeric substrate, and to test such surfaces for blood compatibility in vitro. The investigator will prepare a series of hydrogels of varying water content and test such by standard in vitro coagulation studies. Grafting of hydrophilic mixtures to hydrophobic substrates will be performed.

Major Findings:

Reasonable success has been reported in developing grafted materials and a series of hydrogels of varying water content. They have had inconclusive results from the in vitro testing of blood compatibility.

Proposed Course:

They have been granted a six-month extension to provide time to pursue the question of testing blood compatibility by the insertion of vena cava rings in Rhesus monkeys.

Contractor: Research Triangle Institute

Amount: Approximately $75,000

Title: National Dialysis Registry

Objectives:
To develop a national registry of all patients on hemodialysis.

Major Findings:
The National Dialysis Registry collects data from virtually all centers engaged in dialysis in the United States, and prepares from the data statistical analyses for use by the NIAMD and quarterly reports to all cooperating centers. The data include numbers of patients in center or home dialysis, changes in status of these patients, cause of death in patients that expire, length of time on dialysis, etc. Confidentiality of the center and patient identities is maintained.

During the past year analyses of death by primary cause and multiple decrement life tables have also become available.

The establishment of the Registry, although primarily devoted to vital-statistics fact finding, will allow, should more in-depth be required, the broadest possible base for statistically valid sampling which would not put a large load of data collection on any one center.

Contact is also maintained with the European Transplant and Dialysis Registry, and efforts are being made to make the data on dialysis compatible so that direct comparisons will be possible.

Proposed Course:
To continue obtaining, cataloging, storing, and making available data of patients on dialysis.

Contractor: Rockefeller University

Amount: $42,660

Title: Solute Behavior of Biochemicals Affecting Their Diffusability Through Cellophane Membranes

Objectives:

This new contract will study the basic parameters affecting the dialysis behavior of a wide variety of solutes and improve further the thin film dialysis apparatus. These parameters will include: molecular weight, pH, solvent system, pore size, protein binding, etc.

Contractor: A. J. Sipin Company

Amount: $36,200

Title: Dialysate Delivery System

Objectives:
This contract has as its objectives the development of separate passive fluid resistive elements to meter the flows of the concentrate in water lines on a dialysate delivery system. The pressure drop across the elements is maintained at the same value by a simple concentrate pressuring system. The ratio of the concentrate flow to water flow is in inverse proportion to the resistance of the metering elements.

Major Findings:
Prototypes of this proportioning delivery system have been developed and have been run for some number of hours. The contractor is attempting to maintain a constant flow rate for approximately 10 to 14 hours. This has not yet been accomplished.

Proposed Course:
The contractor will continue to develop this system toward the goal of a continuous delivery of dialysate of predetermined conductivity for at least 10 hours.

Contractor: Southern Research Institute

Amount: $68,700

Title: Electro-Regeneration of Ion-Exchange Resins for Deionizing Water for Hemodialysis

Objectives:

This is a new contract to determine the applicability of the ERID process for preparation of water for use in hemodialysis. To accomplish the above objective, it is planned to design and fabricate an ERID unit specifically for use with hemodialysis, to evaluate the performance of the deionizer, and to determine the usefulness and safety of water prepared with the unit in hemodialysis.

Major Findings:

The group has developed a crude model of the ERID unit and it appears to function well. However, there are several technological problems that remain to be solved.

Proposed Course:

They plan to continue technological development of this unit and will try to make it into a cylindrical unit which should have a higher efficiency.

Contractor: Southwest Research Institute

Amount: $25,300

Title: Skin Interfacing Development

Objectives:
This is a new contract to develop a skin cannula junction which will encourage tissue ingrowth and discourage the formation of a sinus tract and will act as a bacteria barrier.

Major Findings:
The investigator has demonstrated that various velour coatings on the outside of cannulas to induce tissue ingrowth were unsuccessful except for one substance, nylon. All cannulae, except the nylon velour coated ones, were extruded from canines. His explanation is that the direction of the nap on the velour may have some effect on its extrusion.

Proposed Work:
The investigator plans to study the effect of fiber orientation in relation to the skin. He will do this by placing the nap in three directions - directed externally, externally and tangential to the cannula surface. He will place nylon collars on half of each of these three groups. He will use pigs this time rather than canines.

Contractor: Stanford Research Institute

Amount: $78,200

Title: Amino Acids, Peptides and Proteoses in Uremia in Man

Objectives:

To investigate the amino acid, peptide and proteose composition of uremic plasma, urine, and dialysate and relate it to that found in normal individuals.

Major Findings:

The investigators have noted a decreased absorption of tryptophan by the gut and an increased clearance of this and other amino acids by the kidney in uremia.

Proposed Course:

They are planning to study the absorption of other amino acids by direct and indirect methods to determine if this defect is specific for tryptophan. Studies will also be done of renal clearance in moderately advanced uremia to see if amino acid transport is abnormal in the renal tubule early in the uremia syndrome. They are thus trying to elucidate the mechanisms of the depressions of the essential amino acids in uremic patients and to evaluate the therapeutic efficacy of supplementation with essential amino acids in patients being maintained by hemodialysis.

Contractor: University of Texas

Amount: $49,700

Title: Mass Transfer During Dialysis

Objectives:

Using mathematical models and data derived from *in vitro* and *in vivo* studies, determine if and how mass transfer data can be translated into information of physiological and clinical significance for patients undergoing dialysis.

Major Findings:

Computer programs have been written which should enable the investigator to examine events during dialysis occurring at the level of the dialyzer and also to examine the events occurring at the level of the patient. Some *in vitro* amino acid dialysance data have been obtained using several different dialyzers.

Proposed Course:

This contract has been terminated.

Contractor: Tulane University

Amount: $126,000

Title: Studies on the Mechanism of Anemia in Patients with Renal Disease

Major Objectives: The contractors are attempting to examine hematologic parameters in patients with varying degrees of renal function impairment. Particular emphasis is being placed on the study of erythropoietin, and on renal erythropoietic factor.

Major Finding: Eighteen patients have entered this study. Preliminary results indicate that the anemia in these uremic patients consists of a significant degree of marrow depression. A few patients have displayed elevated ESF levels.

Proposed Course:
To continue the study of anemia as it develops over the range of renal excretory function in selected patients with renal disease.

Contractor: University of Utah

Amount: $117,500

Title: New Synthetic Membranes for the Dialysis of Blood

Objectives:
Gain an understanding of the relationship of the molecular structure of a polymer to its membrane diffusion properties and to its surface interactions with blood.

Major Findings:
Characterization of the synthetic block copolymer membranes using a modified Katchalsky approach has been continued. Work has also continued on the interaction of blood constituents and polymer surfaces with emphasis on polymer platelet interactions using the closed cell which was developed by Dr. Lyman for ex vivo investigation. The data developed to date confirmed the direct correlation between platelet adsorption and critical surface tension. Polymer surfaces have been precoated with separate coatings--albumin, gamma globulin, and fibrinogen. The resultant data have demonstrated that albumin-coated polymers tend to be the least thrombogenic from the standpoint of platelet aggregations in the ex vivo test system.

Proposed Course:
Continue investigations in the area of basic mechanisms of membrane transport and the relationship of plasma proteins, free surface energy and platelet adsorption on membrane surface thrombogenicity. His contract proposal was not renewed and he has discontinued all contract work for Artificial Kidney - Chronic Uremia Program.

UNIVERSITY OF UTAH

Amount: $30,000

Title: A Development of Single Needle Dialysis

Objectives:

Objective of this contract is to develop a device whereby a patient can be dialyzed with the use of a single needle puncture into the aterio-venous fistula rather than the currently common practice of using two needles for dialysis. This is a new contract and has just been initiated and there are no significant advances yet made.

Contractor: The University of Utah

Amount: $79,600

Title: Development and Testing of an Efficient and Less Costly Artificial Kidneys for Hospital and Home Use.

Objective:
To develop and evaluate efficient and low cost artificial kidneys for home and hospital utilization.

Major Findings:
1. The artificial control and detection system has been developed and is in clinical use both in the home and dialysis training center.

2. Resterilization of coils. The average cost per dialysis has been reduced from $33 to $22. A study to best define procedures and evaluate the changes in permeability and in clearance of resterilized kidneys is in progress.

3. Nephrophane. This membrane which was investigated over the past year has been found to be not significantly better and in actuality approximately equal to Cuprophane for transport of various solutes. There is a question of increased middle molecular weight molecules with this membrane.

4. Semi-disposable kidney. A new prototype of the dialyzer support system has been developed. This design must still be tested and evaluated.

Proposed Course:
This will include continuing efforts to optimize reuse and resterilization of coils in terms of efficiency of dialysis time, cost, and labor. The semi-disposable membrane to be investigated in a S type kidney utilizing a disposable membrane insert. Investigation will be made into novel compatible surfaces and adsorption kidneys. The proposed work calls for a study of methods of coating the adsorbent with synthetic and natural hydrogels. The contractor will characterize blood compatibility adsorption properties of such coated adsorbents under conditions of importance to the Artificial Kidney - Chronic Uremia Program.

Contractor: Veterans Administration Hospital
Hines, Illinois

Amount: $32,000

Title: Development and Evaluation of a Miniature Parrallel Flow Hemodialyzer

Objectives:

The contractor seeks to fabricate and evaluate clinically the miniature parallel flow hemodialyzer and to improve its performance and reduce the cost of the dialyzer by developing improved membrane supports, flow geometry, and antithrombogenic properties.

Major Finding:

New cone supports have been developed and will be incorporated into the design of the dialyzer.

Proposed Course:

To continue developmental research on improving the efficiency of the miniature parallel flow hemodialyzer.

Contractor: University of Washington

Amount: $26,000

Title: Development of a Low-Cost Home Peritoneal Dialysis System

Objectives:
Develop and build an automatic low-cost peritoneal dialysate system which may be used in the home and will consist of a sterilization tank for preparing dialysate water and then a proportioning pump to provide necessary dialysate constituents prior to infusion to the patient.

Major Findings:
The investigator has been working to perfect the delivery of peritoneal dialysis solution to the peritoneal dialysis patient in the home by the use of a compact machine which he has developed. During this contract year he initiated studies using reverse osmosis as a water purification method rather than distillation. Reverse osmosis has a potential use, but the investigator must still test the R.O. membranes for pyrogen and bacteria rejection. He has used one R.O. unit up to 4 months without bacterial growth. He has perfected the APD (automatic peritoneal dialyzer) so that it should soon be installed in the homes of six patients.

Proposed Course:
The investigator plans to determine the reliability of this system and its place in the home treatment of chronically uremic patients by installing the system in the home of six patients. He is going to do a pilot study on R.O. produced water in humans. He will also evaluate the Osmonics(R) R.O. unit in the preparation of dialysate.

Contractor: The University of Washington

Amount: $20,000

Title: Evaluation of the Dow Hollow Fiber Artificial Kidney for Use in Home Dialysis

Objectives:
This new proposal is designed to undertake broad studies to allow exploitation of full potential of their promising hemodialyzer for home dialysis use.

Major Findings:
This is a new contract which has just recently been initiated. There have been no significant results forthcoming as yet.

Contractor: The University of Washington

Amount: $172,600

Title: Fluid Dynamics Mass Transfer and Optimization Studies of Hemodialyzers

Objectives:
To investigate fluid dynamics and mass transfer characteristics necessary to optimize design and fabricate a hemodialyzer.

Major Findings:
A small 1/3 square meter mini dialyzer has been developed which gives comparable mass transfer as does the larger conventional flat plate dialyzers. It has also been discovered that patients on a low flow, i.e., 100 ml dialysate flow dialysis do as well or better than the patients on 500 ml dialysate flow dialysis. It is hypothesized that this indicates a deficiency syndrome of sorts.

Proposed Course:
Continue investigations of mass transfer and potential ramifications of the "low flow dialysis." Included in these studies are: (1) mass transfer rates of various solutes, (2) clinical status and laboratory status of patients maintained on low flow dialysis, (3) initial studies on the effect of protein binding on various drugs within the patient undergoing dialysis.

Contractor: The University of Washington

Amount: $152,100

Title: Cannula Research for Hemodialysis

Objectives:
Investigate by means of a broad approach improvement of an access to the circulation.

Major Findings:
The sheep has been evaluated as a model for cannula testing. The larger bore AV cannula has been adequately developed and has proven to be useful in that a higher flow can be achieved through it. The Dacron applique shunt has not been used recently because of recurrent infection problems with at least one fatality associated with this. The problems of infection prevention are currently being studied in the Dacron applique shunt. Dipyridamole has been found to apparently inhibit platelet aggregation *in vivo*.

Proposed Course:
Continue the current objectives utilizing the animal shunt model to assess the effects of various designs and drugs on cannula longevity.

Contractor: University of Washington

Amount: $88,700

Objectives:

The objective of the contract is to study the anemia of renal failure by using chronically hemodialyzed sheep as the animal model. The following areas will be investigated: (1) oxygen transport mechanism in the normal and anephric state; (2) the relationship of erythropoietin and marrow production to changes in oxygen delivery in the normal and anephric state; (3) the effect of chronic hemodialysis on erythropoietin function and oxygen transport in the anephric state; (4) the role of inhibition of marrow function in uremic state.

Major Findings:

Preliminary data has been accumulated on the quanitation of the oxygen transport mechanism in the normal state on the relationship of ESF and marrow production to changes in oxygen delivery in normal and anephric state, and on the role of inhibition of marrow function in the uremic state.

Proposed Course:

To continue gathering data on the various factors contributing to the anemia of renal failure.

Contractor: University of Washington

Amount: $74,829

Title: Role of Trace Elements in Toxicity and Treatment of Bone Disease of Maintenance Dialysis Patients

Objectives:

This project is directed at determining whether fluoride therapy will reverse the bone loss seen in patients with uremia and to evaluate whether or not, or to what extent, accumulation of trace metals in bone of uremic subjects plays a role in causing bone disease in uremics. Additionally, the effects of high calcium intake will be studied. Bone biopsies, fluoride analyses, calcium absorption studies, bone density, and trace metal analyses will be performed as well as other biomedical tests.

Major Findings:

Fluoridation of Seattle water, deionizer and other difficulties precluded carrying out the fluoride studies. Preliminary data suggested there was no trace metal accumulation in bone of dialyzed patients. An interesting finding was a delayed but significant absorption of ingested Ca by uremic patients. This contract is being terminated.

Contractor: University of Washington

Amount: $35,387

Title: Peripheral Neuropathy of Uremia

Objectives:

This project will further assess the relationship of nerve conduction time to the adequacy of dialysis and the extent of uremia, and to study the usefulness of electromyography. Correlations will be made with the clinical state and neurological examinations for neuropathy. Necropsy peripheral material will be studied by both light microscopy and electron microscopy.

Major Findings:

It was found that "well-dialyzed" patients showed an improvement in motor nerve conduction velocity into the lower limits of normal. The major difficulty with the test is a large observer variability, resulting in a less than desirable precision. No histologic changes were noted on light microscopy. This contract is being terminated.

INTERNATIONAL RICE RESEARCH INSTITUTE (PH-43-67-726)....U.S.-Japan Program

Amount: $49,000

Title: Improvement of the Protein Content of Rice

Project Director: Dr. B. Juliano

Project Officer: Dr. B. T. Burton (NIAMD)

Objectives:
The primary objective of this project is to determine the feasibility of incorporating a high level protein into commercially produced rice in Southeast Asia. Presently used commercial varieties of rice contain from seven to seven and one-half percent protein. The biological quality of rice protein is fairly high and an increase in the protein level in rice would go far toward alleviating protein undernutrition in countries for whom rice is the mainstay of subsistence. Since 60 percent of the world's population subsists primarily on rice, the implications for an amelioration of the chronic protein shortage in many underdeveloped countries, should this project prove successful, are quite obvious. If the present protein level of rice could be elevated by several percentage points an automatic and sweeping nutritional improvement would take place in major areas throughout the world.

Methods Employed:
The modus operandi involves the screening, for high protein content, of over 7,000 varieties of rice archived in the International Rice Research Institute collection, followed by selective breeding and development of a commercially feasible variety which incorporates an improved protein level together with all the necessary attributes which would make this rice attractive to the average Southeast Asian rice grower, namely high yield per acre, resistance to endemic diseases, strong response to fertilization, and--most important--desirable organoleptic qualities with respect to stickiness, mouth-feel and taste.

Major Findings:
Over 100 rice varieties have been identified among the many thousands tested which contain between 13-1/2 to 17-1/2 percent protein. It is now the purpose of the contract to identify, through actual growing experiments under Southeast Asian climatic conditions, the specific rare high protein varieties (from among these 100 varieties) which would also be agriculturally and commercially feasible in a Southeast Asian setting. Thus far six high protein strains of rice (some from Hungary and some from Southeast Asia) have been successfully crossbred with a new rice variety (IR_8) which has been developed previously at the International Rice Research Institute and which possesses near-ideal plant characteristics including disease resistance and a very high yield per acre. Some of the new hybrid lines have shown as high a protein content as the high-protein parent. Others, of fairly acceptable "plant type" have a protein content of +-11 percent, i.e., contain 50 percent more protein than currently produced commercial rice.

Based on the most recent breeding work involving progeny of successful crosses between the six high-protein strains and IR_8 the project staff is encouraged that the protein content of rice is indeed a genetically determined characteristic. (It has always been known that a variety of environmental factors may be important determinants of the final protein concentration in the rice kernel.) At present the experiments are at the F_5 plant stage (which are making F_6 seed kernels). At this point, the plant breeders are suggesting to back-cross the F_6 generation to IR variety parents which produce greater yields per rice paddy area than the current hybrid plants. While the breeders are waiting for the F_5 plants to mature, the biochemical staff is concentrating on elucidating the metabolic steps or environmental factors in protein biosynthesis which are rate limiting (for instance, in cloudy weather when the sunlight is less strong the protein concentration in rice is enhanced; on the other hand, the total yield decreases because of an increased sterility among the plants).

A seemingly negative correlation between protein concentration and lysine concentration has been observed. Varieties with 15 percent protein content have a somewhat lower lysine concentration in their protein than those containing the more usual 7-1/2 percent protein. At present, Dr. Cruz is collecting the rising sap at the base of the rice panicle and analyzes it for total nitrogen and amino acid composition. Comparisons are made with the amino acid composition found in the final grain in an effort to establish if one amino acid is possibly the limiting factor in final protein concentration. Also, the free amino acids in the sap going into the panicle during the course of the development of the rice grain are being analyzed quantitatively to determine whether differences exist in the level of each free amino acid in low- and high-protein lines. A spectrum of high to low protein-producing rice plants is studied in this fashion. Preliminary data in selected hybrids show that the soluble amino nitrogen concentration and the rate of leucine-U-C^{14} incorporation were higher in developing grains of high protein lines than in low protein ones. Present experiments seek to determine by manipulation of N supply during grain development whether the inherent capacity for protein synthesis is dependent on the level of free amino acids in the grain. If such a relationship exists, then differences in grain protein are mainly due to varietal differences in the efficiency of roots in absorbing N from the soil as reflected in the level of free amino N translocated into the grain.

Protein efficiency ratio (PER) studies, using rats, of several parent rice strains have been carried out by Dr. Bressani at the Institute of Nutrition of Central America and Panama (INCAP) and future PER studies of the new hybrid varieties are contemplated. Results to date have shown that the growth rate of rats was more affected by differences in protein content between the samples than by differences in protein quality.

Dr. Helen Clark of Purdue University has just finished a series of human nitrogen balance studies utilizing one of the experimental high protein rice strains as the source of dietary nitrogen. This strain (BPI 76-1) was compared in adult subjects with an equal weight of rice of the commercial "Bluebonnet" variety at a daily intake of 480 grams. The experimental rice which contained 1.8 times as much protein as Bluebonnet rice, caused

significantly higher nitrogen retention ($P < 0.01$), which is attributable to the increased amounts of essential amino acids. The respective mean positive balances were 1.41 ± 0.89 g and 0.24 ± 0.31 g. Addition of supplementary nitrogen to make the Bluebonnet rice isonitrogenous with the BPI-76-1 variety did not cause a statistically significant improvement. All subjects were in negative balance when 320 g Bluebonnet rice were made isonitrogenous with 480 g of the same rice by adding nonspecific nitrogen.

Significance:

(Please see above under Objectives.) Ordinary commercial rice does not contain enough protein to support normal growth and development of infants and children maintained on a predominant rice diet, and does not protect the adult population from serious protein under-nutrition. Rice is the principal food of 60 percent of mankind. If the present protein level of rice (approximately 7-1/2 percent) could be elevated by several percentage points an automatic and sweeping nutritional improvement would take place in all the populations for whom this one crop represents the nutritional mainstay. Such a development would concomitantly relieve the current burden of the United States which supplies hundreds of millions of dollars' worth of supplementary foods to underdeveloped rice-eating countries. The potential nutritional improvement among Southeast Asian populations and underdeveloped rice-eating countries throughout the world is an important aim of the U.S.-Japan Program and of the applied nutrition program of the Institute.

Proposed Course:

In the face of the encouraging progress thus far, further crossbreeding and growing of the resulting hybrids in large plots is contemplated, side-by-side with additional protein efficiency ratio determinations, in rats, and human nitrogen balance studies, of the protein of the resulting hybrid crops.

FOOD COMPOSITION TABLE FOR USE IN EAST ASIA----U.S.-Japan Program

Amount:
Interagency Agreement with the Nutrition Program, HSMHA: $10,600

Contract for additional personnel with the American Institute of Nutrition: $34,895

Project Director: Dr. W. T. Wu Leung, Nutrition Program, HSMHA

Project Officer: Dr. B. T. Burton, NIAMD

Objectives:
The primary objective of this project is the preparation of a table of food compositions for foods used in the East Asian countries (Burma, Thailand, Laos, Vietnam, Cambodia, Malaysia, Indonesia, Philippines, Taiwan, Korea, and Japan). This includes indigenous food products as well as foodstuffs imported to these countries which are consumed locally. The first Far Eastern Food Table was issued in 1945 under the auspices of the Committee on International Food Value Problems of the National Research Council, U.S.A. Although it was revised and enlarged in 1952 as Agriculture Handbook No. 34, "Composition of Foods Used in Far Eastern Countries", it is not applicable to conditions in 1969. Comprehensive food tables for local foods in East Asia are essential for and basic to any attempt of improving the nutritional state of East Asian populations through a combination of nutrition education or nutrition improvement through introduction of innovations in the local food consumption.

Methods:
The Principal Investigator, Dr. Leung, who was responsible for the preparation of the first Far Eastern Food Table immediately after World War II, is collecting all available food data through correspondence and through personal visits in the various countries. In addition, there is active collaboration with the Nutrition Division of the Food and Agriculture Organization of the United Nations, through Dr. K.K.P.N. Rao and his staff who have accumulated a considerable body of data at FAO Headquarters in Rome and are constantly generating new information in regional FAO offices in Asia.

The information collected includes: data on moisture, "proximate composition", minerals, vitamins, amino acids, fatty acids, trace elements, refuse, and local methods of preparation, processing and preservation of foods, etc. Whenever feasible, local names used in various Far Eastern countries with the corresponding English and scientific names are recorded and collected. The result of this project will be printed as a joint publication "Food Composition Table for Use in East Asia" - NIAMD will be responsible for the printing of the English version, and FAO will undertake the translation and printing in French. The format of the food table will follow closely the one previously designed for Africa.

Major Findings:
Data collection was begun in the winter of 1969-70 and is progressing very satisfactorily.

Significance:
Please see "Objectives".

Proposed Course:
It is expected that data collection will proceed for about two years, while simultaneously, tables will be coordinated and assembled in Bethesda. A third year will be necessary for the final organization, editing and printing of the publication.

THE ANNUAL REPORT SUMMARY
EPIDEMIOLOGY & FIELD STUDIES BRANCH

The major interests of the Branch have remained in the fields of arthritis, diabetes mellitus, gallbladder disease, and goiter.

SOUTHWESTERN FIELD STUDIES SECTION

Dr. Thomas A. Burch, Formerly Chief of the Southwestern Field Studies Section, received the Meritorious Service medal for his long and distinguished service in the Public Health Service.

In December, 1970, Doctor Burch received an assignment as Chief of the Office of Research and Statistics with the State Health Department of Hawaii. Doctor Burch retired from the Public Health Service on May 31, 1971. Dr. Peter H. Bennett has served as Acting Chief of the Section since December, 1970.

The Southwestern Field Studies Section, located in Phoenix, Arizona has continued to have its major interest in the Pima Indian population, residing on the Gila River Indian Reservation. In this well defined population studies of arthritis, diabetes mellitus and gallbladder disease have continued.

Gallbladder Disease

For the first time in any population the true prevalence of gallbladder disease was described. It was found that among the Pima Indians gallbladder disease is extraordinarily frequent in both males and females, and that less than one half of the affected subjects had been recognized clinically. When the true prevalence was defined it became apparent that only a very incomplete picture of the distribution of the disease was ascertained through clinical diagnoses. The disease among females was found to have an abrupt onset between the ages of twenty-two and twenty-seven years, with the prevalence reaching seventy percent at the latter age.

When carefully controlled observations were made the purported associations of gallbladder disease with obesity, diabetes mellitus, number of pregnancies and serum cholesterol levels were found to be absent.

These findings led other workers to make studies on the pathophysiology of gallbladder disease among the Indian people. It has now been determined that

a) American Indians with cholelithiasis have mainly cholesterol gallstones, as do non-Indians.

b) American Indians with cholelithiasis secrete hepatic bile which is supersaturated with cholesterol. This finding has also been confirmed in non-Indians.

c) Young American Indian women who have not yet developed gallstones frequently have bile which is supersaturated with cholesterol also.

d) The composition of the abnormal bile may be converted to that of an unsaturated solution by the oral administration of cheno-deoxycholic acid, a normal bile salt.

Studies of the natural history of gallbladder disease among the Pima Indians have also indicated that among subjects with gallstones of presumed recent onset the gallbladder retains its concentrating ability so that the cholecystogram shows a visualizing gallbladder containing stones. Later the gallbladder becomes non-functioning, so that it becomes non-visualizing on cholecystogram. Studies in subjects coming to cholecystectomy showed that the subjects with visualizing gallbladders have relatively few complications and infrequent evidence of chronic inflammation, whereas those with non-visualization usually had chronic inflammatory changes and many clinical complications.

These findings are consistent with the hypothesis that the basic abnormality in gallbladder disease is a primary metabolic defect leading to the secretion, by the liver, of bile of abnormal composition and supersaturated with cholesterol. Precipitation of the cholesterol occurs in the gallbladder and crystal growth then leads to gallstone formation. The presence of gallstones in the gallbladder leads to chronic inflammatory changes and to the many complications which result in clinically manifest gallbladder disease.

This increased understanding of gallbladder disease in American Indians strongly suggests that gallbladder disease is preventable if susceptible subjects were identified and if their bile composition was normalized. It may also be possible to dissolve gallstones by changing bile composition by medical methods and thus preventing many or all of the clinical complications of the disease.

Further studies of the nature of the basic metabolic defect and possible curative and preventative methods will be continued in collaboration with the newly formed Phoenix Clinical Research Section.

Diabetes Mellitus

The studies of diabetes mellitus conducted by the Southwestern Field Studies Section have emphasized two areas, the possible cause of the basic abnormality and factors contributing to the specific vascular complications of diabetes mellitus.

Insulin levels among Pima Indians have been examined to determine whether the bimodality observed in distributions of post load glucose levels is related to the insulin level. It was found that subjects who formed the

hyperglycemic subpopulation characteristically had low serum insulin levels, and that those with normal glucose levels had insulin levels which were proportional to the glucose level. This finding, in conjunction with the observation that the specific vascular complications occur only in the hyperglycemic subjects, supports the view that true diabetes mellitus is characterized by hypoinsulinemia.

The mechanism of onset of glucose intolerance was explored by determination of antibody titers to Coxsacke B4 virus, an agent known to cause pancreatitis in experimental animals. It was found that a significantly greater proportion of subjects who had recently developed marked glucose intolerance had titers which indicated a recent infection than did carefully matched control subjects. This finding confirms a similar report by British workers and indicates the need for much more work concerning the possible viral etiology of diabetes mellitus.

The vascular complications of diabetes mellitus, especially retinopathy and nephropathy, have been found almost exclusively among Pima Indians with two hour post load glucose levels in excess of 200mg/100ml. Within this group, however, little or no relationship of prevalence of complications to glucose level was found. This finding suggests that diabetes mellitus is qualitatively a discrete disease and that the absolute level of glucose intolerance is not related to frequency of development of the specific complications.

Diabetic retinopathy within the Pima Indian population shows the same associations as have been described in other non-Indian diabetics, but retinopathy was also found to be associated with elevated levels of systolic and diastolic blood pressure and serum cholesterol. These observations may have important pathophysiologic significance and if these variables can subsequently be shown to be causally related to retinopathy important developments in the treatment and prevention of retinopathy may follow.

Renal disease has been shown to be a very common complication of diabetes among the Pima Indians and as very little is known concerning the natural history and early characteristics of this complication, detailed prospective observations of susceptible subjects will be made to define the earliest stages of the disease.

METABOLIC DISEASES EPIDEMIOLOGY UNIT

The role of contaminated water supplies in the pathogenesis of endemic goiter have been investigated by the Metabolic Diseases Epidemiology Unit. Several bacterial species have been shown to be associated with some goitrogenic activity. Further studies to purify and isolate the active agents are in progress at this time.

Serial No. NIAMD-EFS-1(c)
1. Epidemiology & Field Studies Br.
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Prevalence of Diabetes in Indian Populations in the Southwestern United States

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Thomas A. Burch
Mr. Gregory Bartha

Cooperating Units: Indian Health Service (PHS)

Man Years:

Total:	0.6
Professional:	0.3
Other:	0.3

Project Description:

Objectives:

1. To determine the prevalence of diabetes among various native populations in the Southwestern United States.

2. To formulate and test hypotheses which may explain the high frequency of diabetes mellitus among some of these populations.

Methods:

Random samples of total populations will be examined by means of standardized tests of glucose tolerance to enable the prevalence of glucose intolerance to be determined. Other examinations will be performed to document the frequency of other disorders when appropriate.

Major Findings:

A survey was conducted to determine the frequency of glucose intolerance among the Washoe and Northern Paiute tribes residing in Nevada. Both tribes reside in similar environments. The Washoe are linguistically related to the Yuman Indians and the Cocopah and the Paiute to other Uto-Aztecan groups such as the Pima.

The prevalence among the two tribes did not vary significantly, but both had prevalences of hyperglycemia, which were significantly lower than among the Cocopah and Pima Indians. The rates found were higher than previously found among the White River Apache.

PREVALENCE OF HYPERGLYCEMIA* AMONG SOUTHWESTERN INDIANS

	Males				Females			
Age (years)	<35		>35		<35		>35	
Tribe	No. Exam.	Percent Positive	No. Exam.	Percent Positive	No. Exam.	Percent Positive	No. Exam.	Percent Positive
White River Apache	--	--	109	3.7	--	--	159	18.2
Washoe	16	0.0	34	8.8	30	3.3	32	25.0
Paiute	39	0.0	27	18.5	38	2.6	27	33.3
Upland Yumans	52	0.0	144	21.2	93	1.8	169	38.6
Zuni	87	2.9	140	17.2	312	3.8	152	45.4
Cocopah	15	6.7	35	25.7	29	6.9	44	40.9
Pima	921	4.3	437	41.0	1098	6.5	461	58.1

*Plasma glucose level ≥160mg/100ml two hours post 75G carbohydrate load or ≥180mg/100ml one hour post load.

Significance of Research:

The prevalence rates of diabetes mellitus among the various Indian Tribes in the Southwestern United States so far studied appear to be substantially unrelated to their genetic origins. This finding suggests that the high prevalence of diabetes observed in many may represent an adaptive mechanism with a genetic basis to certain types of environment rather than simply an expression of a common genetic background.

Proposed Course:

Short term studies of other tribes living in the Southwestern United States will be conducted to increase knowledge of the distribution of diabetes mellitus when feasible.

Honors and Awards: None

Publications: None

Serial No. NIAMD-EFS-2(c)
1. Epidemiology & Field Studies Br.
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Prospective Study of the Natural History of Diabetes Mellitus in the Gila River Indian Community

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Thomas A. Burch
Dr. Arthur Dorf
Dr. Max Miller
Dr. Arthur G. Steinberg
Dr. Norman B. Rushforth
Dr. Elmer J. Ballintine
Dr. Irving M. Liebow
Dr. Frederick A. Rose
Dr. James E. Maynard
Dr. Jesse Roth
Dr. Phillip Gorden

Cooperating Units:
Indian Health Service (PHS)
Case Western Reserve University
Center for Disease Control (PHS)

Man Years:

Total:	8.5
Professional:	2.5
Other:	6.0

Project Description:

Objectives:

1. Determine the prevalence and incidence of chemical and clinical diabetes mellitus among those five years of age and over on the Gila River Indian Reservation.

2. Evaluate factors associated with the progression from prediabetic state to chemical diabetes mellitus and to clinical diagetes.

3. Study the association between diabetes and rate of appearance and progression of vascular complications.

4. Investigate influence of tribe, age, obesity, parity, and other factors on changes in glucose tolerance and their relationship to diabetes mellitus.

Methods:

1. The following tests and examinations will be repeated on all Indians five years of age or more living on the Sacaton Service Unit portion of the Gila River Reservation:

a. Clinical interview
b. Physical examination
c. Modified glucose tolerance test
d. Fundus photographs
e. Electrocardiogram
f. Tests for vibratory sensation threshold
g. Soft tissue radiographs for vascular calcification

2. Some of the above tests and examinations will also be done on Indians of other tribes or living under different environmental conditions for comparison.

Major Findings:

1. Retinopathy: Fundus photographs and funduscopic examinations were performed on 1,147 Pima Indians, aged 15 and older. A modified glucose tolerance test, consisting of a 75 gm oral carbohydrate load followed by a venous plasma glucose determination two hours later, was given to each subject. Without knowledge of the results, elements of retinopathy were separately recorded.

Exudates were most prevalent, followed in order by microaneurysms, preretinal and vitreous hemorrhages, and proliferative changes and neovascularization in both males and females.

PERCENT OF SUBJECTS WITH RETINAL ABNORMALITIES

	Males		Females		Both Sexes
	No. Exam.	Percent Positive	No. Exam.	Percent Positive	Percent Positive
Microaneurysms	509	6.5	638	6.9	6.7
Exudates	509	7.3	638	9.9	8.7
Preretinal and vitreous hem.	509	2.0	638	1.9	1.9
Prolif. and neovascular changes	509	0.6	638	0.9	0.8
One or more of the above elements of retinopathy	509	10.4	638	12.1	11.3

All elements of retinopathy were more frequent among those with elevated two hour plasma glucose levels, especially among those with levels in excess of 200mg/100ml.

PERCENT OF SUBJECTS WITH ONE OR MORE ELEMENTS OF RETINOPATHY AND THEIR RELATIONSHIP TO PLASMA GLUCOSE LEVELS

Two Hour Plasma Glucose (mg/100ml)	Males No. Exam.	Males Percent Positive	Females No. Exam.	Females Percent Positive	Both Sexes Percent Positive
400+	43	34.9	72	36.1	35.6
300-399	39	23.1	41	43.9	33.8
200-299	27	25.9	58	13.8	17.6
100-199	257	6.2	347	5.8	6.0
000-099	143	4.2	120	4.2	4.2

The prevalence of retinopathy was related to the duration of diabetes. Some 60 percent of those with duration of ten years or more had one or more elements of retinopathy compared to about 10 percent of those who had diabetes for less than one year.

PERCENT OF SUBJECTS WITH ONE OR MORE ELEMENTS OF RETINOPATHY AND THEIR RELATIONSHIP TO DURATION OF DIABETES

Duration of Diabetes	Males No. Exam.	Males Percent Positive	Females No. Exam.	Females Percent Positive	Both Sexes Percent Positive
Less than 1 year	7	14.3	6	0.0	7.7
1-4 years	29	30.6	64	25.7	27.4
5-9 years	23	34.8	36	27.8	30.5
10 years or more	17	41.2	33	66.7	58.0

2. Blood Pressure and Retinopathy. With increasing systolic and diastolic blood pressure retinopathy was increasingly prevalent. The positive gradient was discernable even over the normal range of diastolic blood pressure.

PERCENT OF SUBJECTS WITH ONE OR MORE ELEMENTS OF RETINOPATHY AND THEIR RELATIONSHIP TO SYSTOLIC BLOOD PRESSURE

Systolic B.P. (mmHg)	Males No. Exam.	Males Percent Positive	Females No. Exam.	Females Percent Positive	Both Sexes Percent Positive
200-280	7	14.3	16	56.2	43.5
180-199	20	25.0	23	43.5	34.9
160-179	53	22.6	48	37.5	29.7
140-159	119	11.8	109	15.6	13.6
120-139	231	6.1	225	4.0	5.0
100-119	74	9.5	191	5.8	6.8
080-099	4	0.0	26	11.5	10.0

PERCENT OF SUBJECTS WITH ONE OR MORE ELEMENTS OF RETINOPATHY AND THEIR RELATIONSHIPS TO DIASTOLIC BLOOD PRESSURE

Diastolic B.P. (mmHg)	Males No. Exam.	Males Percent Positive	Females No. Exam.	Females Percent Positive	Both Sexes Percent Positive
120-140	4	50.0	1	100.0	60.0
100-119	44	18.2	31	32.3	24.0
080-099	196	14.8	205	17.1	16.0
060-079	225	5.8	340	8.5	7.4
040-059	37	2.7	58	3.4	3.2
000-039	2	0.8	3	0.0	0.0

The population sample from which this information was derived contained diabetic and nondiabetics, over a wide age range, with and without renal impairment. To eliminate these variables, a specific sample was selected. Among 145 diabetics, aged 35-64, with normal renal function, retinopathy was found in about one-fifth of those with systolic blood pressure <140 mmHg, in one-third of those with systolic blood pressure of 140-159 mmHg, and in over one-half of those with systolic blood pressure ≥160mmHg. This relationship was significant even after adjustment for duration of diabetes ($p<0.05$).

PERCENT OF SUBJECTS WITH ONE OR MORE ELEMENTS OF RETINOPATHY

Systolic B.P. (mmHg)	No. Exam.	No. Positive	Percent Positive
Less than 140	59	10	17.0
140-159	43	14	32.6
160-179	33	18	54.6
180+	10	6	60.0

The prevalence of retinopathy was also related to the serum cholesterol level. As with diastolic blood pressure, a positive relationship was found even over the normal range. The normal maximum serum cholesterol for the Pima Indian population is approximately 250mg/100ml.

PERCENT OF SUBJECTS WITH ONE OR MORE ELEMENTS OF RETINOPATHY

	Males		Females		Both Sexes
Serum Cholesterol (mg/100ml)	No. Exam.	Percent Positive	No. Exam.	Percent Positive	Percent Positive
280+	16	31.2	16	37.5	34.4
240-279	35	25.7	42	21.4	23.4
200-239	100	15.0	120	17.5	16.4
160-199	166	7.2	230	12.6	10.4
120-159	149	7.4	179	5.0	6.1
080-119	38	2.6	39	5.1	3.9

3. Renal Disease. Evidence of renal disease and its relationship to glucose intolerance was examined among Pima Indians age 35-74 years.

PERCENT OF SUBJECTS WITH PROTEINURIA

	Males			Females		
Glucose Level (mg/100ml)	No. Exam.	Percent w/proteinuria ≥30mg/100ml	≥1g/day	No. Exam.	Percent w/proteinuria ≥30mg/100ml	≥1g/day
50-99	47	6.4	0.0	17	11.8	4.9
100-125	55	5.5	1.8	61	4.9	0.0
126-159	37	10.8	0.0	54	1.9	0.0
100-199	20	15.0	5.0	39	7.7	0.0
200-250	11	18.2	18.1	19	21.1	11.8
251-399	26	19.3	11.1	56	21.3	12.5
400+	22	18.1	18.2	61	23.0	18.3

It was found that proteinuria and in particular heavy proteinuria was common among those with two hour post load glucose levels in excess of 100mg/100ml, but beyond this level the degree of glucose intolerance was

unassociated with the frequency of proteinuria.

4. Insulin Levels and Their Relationship to Plasma Glucose Levels. Serum insulin (SIRI) and venous plasma glucose levels were determined 0, 30, 60 and 120 minutes after a 75 gram oral carbohydrate load among 230 Pima Indians who had previous two hour glucose levels of ≥140mg/100ml.

Subjects with two hour glucose levels upon retesting of ≤140, 141-200, 209-300 and 301-400, and over 400mg/100ml had mean two hour SIRI levels (± SEM) of 107 (± 9), 150 (± 9), 133 (± 10), 78 (± 7) and 38 (± 3) and μU/ml respectively. The distributions of the SIRI levels showed that among those with glucose levels ranging up to approximately 200mg/100ml, there was an increasing SIRI with increasing glucose level. At higher levels the SIRI was lower, and in those with glucose levels above 300mg/100ml, there was, with rare exception, hypoinsulinemia.

Obesity was associated with somewhat higher two hour SIRI levels for a given glucose level, but its effect was less than the variation related to the plasma glucose level.

Serial glucose and SIRI determinations in over 30 subjects, at approximately two yearly intervals, suggested that in a given subject increases in the two hour plasma glucose level, up to about 200mg/100ml, were accompanied by an increase in SIRI level whereas in those with higher glucose levels, a decrease in insulin level was generally observed.

5. Coxsackie B4 Virus Studies. Thirty-four pairs of sera were identified from subjects before and after the onset of diabetes. Control sera from subjects who had not developed diabetes, matched for age, sex, and date of collection, were used for comparison. Coxsackie B4 antibody titers were determined on both sera in 21 of these pairs and in each of the other 13 pairs. Nine individuals had titers greater than 1:32. Of these nine individuals seven were newly diagnosed diabetics. Thus an excessive number of individuals with high titers of antibodies to Coxsackie B4 virus was identified among the new diabetics ($p<0.05$). Among the 21 matched pairs six of the seven with titers >1/32 were among the new diabetics ($p<0.05$). At this time more paired sera are being identified in an attempt to extend the study.

Significance of Research:

A unique approach to the epidemiology of retinopathy in a total population has demonstrated the relative frequency of each element and that there are associations of increased prevalence of retinopathy with elevated plasma glucose, elevated systolic blood pressure, elevated diastolic blood pressure, elevated serum cholesterol and increasing duration, but not age at diagnosis of diabetes. If the relationships of blood pressure and serum cholesterol prove to be causally related to retinopathy, it seems likely that retinopathy may be preventable using medications currently in use for other purposes.

The development of proteinuria and diminishing renal function among diabetics is well recognized, but the natural history of the disorder and factors affecting the prognosis are poorly understood. The identification of such a large number of subjects with the disease who are being followed at biennial intervals should increase knowledge of the natural history of the disorder. A more detailed biochemical, immunologic and pathologic investigation of this disorder in the Pima Indians is being conducted.

The data concerning insulin levels indicate that Pima Indians with unequivocal diabetes mellitus are characterized by hypoinsulinemia, but that hyperinsulinemia is a characteristic of subjects with high normal or mildly abnormal glucose tolerance. The data emphasized the importance of careful assessment of the degree of glucose intolerance if SIRI levels among subjects with "diabetes" are to be interpreted appropriately.

The finding that Coxsackie B4 antibodies are related to the onset of diabetes mellitus confirms a recent report of Gamble and co-workers (British Med. J. 3, 627, 1969), which suggests that elevated levels of antibodies to the Coxsackie B4 virus are found in recently diagnosed diabetics. Confirmation of this finding has definite pathogenetic implications since this organism is known to be capable of producing a pancreatitis and a closely related picornovirus produces experimental diabetes in mice.

Proposed Course:

The prospective study of diabetes among the Pima Indians will continue. Other factors which are associated with the appearance of the specific vascular complications will be sought.

The course and development of glucose intolerance and concomitant events will be continued and possible changes prior to the development of glucose intolerance will be investigated. Further studies of the possible role of viruses in the etiology of diabetes will be conducted, and periodic analyses to determine the possible modes of inheritance of diabetes will be made.

More detailed studies of the significance of blood pressure, serum cholesterol and other lipids in the pathogenesis of diabetic retinopathy will be performed.

The natural history of diabetic nephropathy will be further investigated by documentation of the qualitative and quantitative patterns of protein excretion and an attempt will be made to identify the earliest stages of this complication.

Honors and Awards: None

Publications:

Levine, S.B., Sampliner, R.E., Bennett, P.H., Rushforth, N.B., Burch, T.A., and Miller, M.: Asymptomatic Parotid Enlargement in Pima Indians: Relationship to age, obesity and diabetes mellitus. Ann. Int. Med. 73, 571-573, 1970.

Bennett, P.H., and Miller, M.: Diabetes Mellitus in Indians of the Southwestern United States. In Rodriguez R.R.(ed): Proceedings VII Congress of the International Diabetes Federation, 1970 Amsterdam Excerpta Medica, In press.

Bennett, P.H., Miller, M., Burch, T.A., Ballintine, E.J., LeCompte, P.M.: Hyperglycemia and the Specific Retinal and Renal Complications of Diabetes in the Pima Indians. In Rodriguez R.R. (ed). Proceedings VII Congress of the International Diabetes Federation, 1970, Amsterdam Excerpta Medica, In press.

Rushforth, N.B., Bennett, P.H., Steinberg, A.G., Burch, T.A., and Miller, M.: Diabetes in the Pima Indians. Evidence of bimodality in glucose tolerance distributions. Diabetes 1971, In press.

Serial No. NIAMD-EFS-3(c)
1. Epidemiology & Field Studies Br.
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Prospective Study of the National History of Arthritis and Rheumatism in the Gila River Indian Community

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Thomas A. Burch
Dr. Arthur Dorf
Dr. Michael J. Shack
Dr. William W. Gough
Dr. Alan L. Metzger
Dr. J. P. Gofton

Cooperating Units: Indian Health Service (PHS)
University of British Columbia, Vancouver, B.C., Canada

Man Years:

Total:	3.5
Professional:	0.5
Other:	3.0

Project Description:

Objectives:

1. Determine the progression of clinical, radiological and serological features of joint diseases affecting the Gila River Indians with particular attention to rheumatoid arthritis, osteoarthrosis, ankylosing spondylitis and gout.

2. Determine environmental and genetic factors associated with development and progression of arthritis and rheumatism.

3. To compare and contrast the clinical course of rheumatoid arthritis in sero-positive individuals with that of sero-negative arthritics.

4. Determine the predicative and prognositic significance of rheumatoid factor in relation to the clinical course of rheumatoid arthritis.

Methods:

1. The following tests and examinations will be repeated on all Indians five years of age or more living on the Sacaton Service Unit portion of the Gila River Reservation.

a. Clinical interview.
b. Physical examination.
c. Radiographs of hands, feet, and cervical spine. Males over age 14 and females over age 44 will have a pelvis film taken.
d. Laboratory determinations: rheumatoid factor, uric acid.

2. The data from the above examinations and tests as well as demographic, pedigree, environmental information, and data on other conditions and diseases will be entered into computer data files for subsequent analyses.

Major Findings:

Sacroilitis. The prevalence of sacroilitis and spondylitis has been compared in the Pima Indians and several northwest coast Canadian Indians.

PREVALENCE OF SACROILITIS IN MALES

	Aged 15 and over		Aged 20 and over	
	No. Exam.	Definite Sacroilitis Percent	No. Exam.	Definite Sacroilitis Percent
Haida	209	8.1	175	8.0
Bella Bella		Not examined	158	7.6
Bella Coola	141	3.2	109	4.6
Pima	223	3.1	166	4.2

The Pima Indians were shown to have a significantly lower prevalence of sacroilitis than the Haida Indians among the males aged 15 years and over. Other differences seen in the table were not statistically significant.

Significance:

The findings reported have confirmed an earlier suspicion that significant differences in prevalence of sacroilitis did exist between the Haida and Pima Indians. The reasons for this difference remain uncertain, but the similarity between the Haida and Bella Bella prevalence, and Bella Coola and Pima prevalences suggest that environmental factors may be important in determining the prevalence rather than solely genetic differences between the populations.

Proposed Course:

The collection of data from the Pima Indians at biennial intervals will continue so that the development and progression of various rheumatic diseases may be observed.

The radiographs collected over the past five years have been evaluated on a joint by joint basis and these readings will be subjected to "pattern" analysis to determine if previously unrecognized syndromes of degenerative and erosive arthritis can be identified.

Honors and Awards: None
Publications: None

Serial No. NIAMD-EFS-4(c)
1. Epidemiology & Field Studies Br.
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Gila River Indian Community Gallbladder Study

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Thomas A. Burch
Dr. William W. Gough
Dr. Alan L. Metzger
Dr. Donald M. Small
Dr. John S. Fordtran

Cooperating Units: Indian Health Service (PHS)
Boston University
Southwestern Medical School

Man Years:

Total:	1.5
Professional:	0.5
Other:	1.0

Project Description:

Objectives:

1. To determine the age and sex-specific prevalence of cholelithiasis in a random sample of Pima Indians living in the Sacaton Service Unit area of the Gila River Indian Reservation.

2. To identify personal and environmental factors associated with cholelithiasis.

Methods:

1. Fifty male and fifty female Pima Indians in each of the six decades between 15 and 74 were selected using random numbers from the NIAMD Demographic Survey of the Gila River Indian Reservation.

2. The Sacaton Hospital records of all selected subjects were reviewed for history of previous cholecystograms or gallbladder surgery.

3. A cholescystogram was performed on all respondents without specified contraindications.

Major Findings:

PREVALENCE OF GALLBLADDER DISEASE IN PIMA INDIANS

	Age Group (Yr)	No. in Sample	No. with Clinical Diagnosis	No.* Exam.	No. With Gallbladder Disease**	Estimated Prevalence (%)
Males:	15-24	45	0	17	0	0
	25-34	45	2	16	0	4.0
	35-44	51	0	27	3	11.1
	45-54	51	2	24	7	31.9
	55-64	47	9	24	14	66.3
	65+	57	5	34	22	67.8
Females:	15-24	45	1	28	3	12.7
	25-34	47	12	25	16	73.2
	35-44	53	22	24	12	70.8
	45-54	47	20	19	11	75.8
	55-64	50	12	26	13	62.0
	65+	58	23	23	19	89.5
Totals:		596				48.6

*No. with cholecystogran + no. autopsied.
**No. with abnormal cholecystogram + no. with cholelithiasis at autopsy.

The prevalence of gallbladder disease in the Pima sample was 48.6 percent. Gallbladder disease was significantly more common in females than in males ($p<0.001$). In females the prevalence rose rapidly to 73 percent in the group from 25 to 34 years of age. In males the prevalence rose significantly after the age of 44 and reached to over 60 percent in the group from 55 to 64 years of age, a rate similar to that in females of the same age.

Determination of gallbladder status by clinical diagnosis alone seriously underestimated the true prevalence. Less than a third of the males and about half the females with gallbladder disease had a previous clinical diagnosis. The proportion of males with gallbladder disease identified by cholecystography was significantly greater than that of females ($p<0.01$).

Of the 116 subjects with abnormal cholecystograms, 37 percent had cholelithiasis, and 63 percent nonvisualization. Sixteen percent of those with cholelithiasis had radiopaque stones. Nonvisualization was significantly more common in males than in females and in the older age groups of both sexes.

The prevalence of gallbladder disease was significantly higher in females than in males and increased with age in both sexes. The age-specific prevalence in females demonstrated an extremely sharp rise between 20 and 25 years of age suggesting that the disease in females usually starts in this

age range. In fact, the mean age at diagnosis among the 13 females less than 35 years old who had previous clinical disease was 24.5 years, with only one diagnosis made before the age of 20. Among all subjects with previous clinical disease, the median age at onset was 26 years in females and 46 years in males. Because 73 percent of the females 25 to 34 years old had gallbladder disease, those 15 to 20 years old were identified as likely to acquire the disease in the ensuing five to 10 years.

The previously purported associations between gallbladder disease and obesity, serum cholesterol level, diabetes and parity were not confirmed in the present study.

The true prevalence of gallbladder disease in other populations has not been established. The estimates available from autopsy studies suggest that the prevalence is much lower in other ethnic groups than in the Pima Indians. The Chippewa tribe has also been shown to have similar high age-specific prevalence rates of clinically diagnosed gallbladder disease. These findings in two culturally distinct tribes suggest that a high prevalence of the disease is characteristic of American Indians. The apparent difference between American Indians and other racial groups may indicate that genetic factors are responsible for the disease.

Autopsy Prevalence of Gallbladder Disease. The autopsy reports available on Pimas from the study population were reviewed. There were reports on 68 males and 54 females over the age of 14 years. Though the number of reports is small the findings are very similar to those reported in the clinical and radiographic survey. The percent with gallbladder disease (GBD) includes those with gallstones at autopsy plus those with surgically absent gallbladders.

PERCENT OF SUBJECTS WITH GALLBLADDER DISEASE AT AUTOPSY

	Males		Females	
Age Group	No. of Autopsies	Percent with GBD	No. of Autopsies	Percent with GBD
15-24	0	-	0	-
25-44	6	17	11	64
45-64	29	45	22	86
65+	34	46	21	71
Total	68	49	54	76

Age at Menarche in those with Gallbladder Disease. The age of menarche was determined for those Pima women known to have gallstones as well as for women who had normal cholecystograms. The age group 25-44 years was chosen since they were old enough to have developed stones and presumably young enough to remember their age at menarche. The mean age at menarche was 12.5 ± 0.2 (SEM) for those with stones and 11.8 ± 0.2 for those without stones. Thus the mean age of menarche was older for those women who developed gallstones ($p < 0.025$).

Relationship of Age and Symptoms to Cholecystographic Findings in Cholelithiasis. The rapid onset of cholelithiasis among Pima women plus the availability of cholecystograms on a large number of normal, clinically silent and clinical cases allows a unique opportunity to study the natural history of gallstone disease. Cholecystograms were classified into three broad categories; normal, visualizing with stones evident, and nonvisualizing. An increasing percentage of the individuals with gallbladder disease developed nonvisualization with increasing age, and clinical cases were more likely to show nonvisualization than subclinical cases. These findings are statistically highly significant. An implication of these findings is that there is a normal progression in cases of cholelithiasis from the normally visualizing state to one of visualization with filling defects, followed in time by the development of nonvisualization.

Relationship of Cholecystographic and Surgical Findings. When both cholecystographic reports and operative notes were available these findings were compared in the female subjects. There were 66 cases with visualization and 72 cases with nonvisualization. Cystic duct blockage, gallbladder fistula, empyema, shrunken atropic gallbladder and common duct stones were reported in only 22.7 percent of those who had had visualizing gallbladders with stones. Similar findings were described in 76.4 percent of those who had nonvisualization of the gallbladder. Conversely the gallbladder was simply described as "containing stones" in 77.3 percent of the cases with visualization but in only 23.6 percent of the cases of nonvisualization. Thus nonvisualization was associated with more advanced gallbladder pathology, as would be expected with a natural progression from an initial state in which the gallbladder visualizes in spite of stones to one of nonvisualization with more advanced disease.

Familial Aggregation of Gallbladder Disease. An attempt was made to determine whether gallbladder disease showed familial aggregation. Forty-two subjects with cholelithiasis and a similar number of age-sex matched controls with normal gallbladders were selected. The presence or absence of cholelithiasis among their first degree relatives was determined by review of their medical histories or cholecystograms if available.

It was found that no information was available on 70-80 percent of the parents and that the majority of their children were of insufficient age to have developed the disease. Further analysis was therefore restricted to siblings (males aged 45 years and over and females aged 25 years and over), 40 percent of whom had adequate data.

CHOLELITHIASIS IN SIBLINGS OF SUBJECTS WITH AND WITHOUT CHOLELITHIASIS

Propositus	With Cholelithiasis		Without Cholelithiasis	
Relatives	No. Exam.	With G.B. Disease	No. Exam.	With G.B. Disease
Males	9	6	10	2
Females	19	17	18	14
Total	28	23 (82%)	28	16 (57%)

Chi square = 4.1 $p<0.05$.

A modest degree of familial aggregation was demonstrated, which may be the result of genetic or environmental factors, or the result of incomplete data on all subjects.

Age and Prognosis in Subclinical Gallbladder Disease. A large number of subclinical or asymptomatic cases of gallbladder disease were discovered during the survey of the prevalence of this disorder. Most of these individuals denied symptoms referable to the gallbladder. During the one to four year follow up period none of the 40 subclinical cases in males are known to have come to cholecystectomy. Nine of the 73 women with follow up data have come to cholecystectomy. Six of the nineteen cases of subclinical GBD found in women aged less than 35 eventuated in interim cholecystectomy, but only three of the 54 cases over the age of 35 have come to cholecystectomy. This greater incidence of cholecystectomy in younger subclinical cases was statistically significant(chi square - 8.85, $p<0.01$).

Significance of Research:

Abnormal hepatic bile, highly supersaturated with cholesterol, has recently been demonstrated in Southwestern American Indians with cholelithiasis. This indicates that gallbladder disease may have a primary metabolic basis. It has also been shown that duodenal bile in a number of Indian females with normal cholecystograms had significantly lower ratios of bile acid and lecithin to cholesterol than that of white females. As the lithogenic potential of such bile appears to be reduced by the oral administration of chenodeoxycholic acid, it may be possible to develop medical methods to prevent gallstone formation in susceptible subjects.

Because of the extremely high prevalence of gallbladder disease, especially in the females, and because of the apparent extraordinarily high incidence in females between 20 and 25 years of age, the Pima Indians form a valuable population in which to examine further the nature of the metabolic abnormality which leads to cholelithiasis. Furthermore, prevention of cholelithiasis by means of alteration of the bile composition appears to be a realistic goal, and the Pima Indians are an ideal population in which to perform studies to develop and assess methods to achieve this aim.

As the precise nature of the metabolic defect is unknown a search for familial aggregation of gallbladder disease is worthwhile and the high prevalence makes the Pima population suitable for such a study.

Proposed Course:

1. The possible familial aggregation of gallbladder disease will be investigated. The prevalence of cholelithiasis will be determined among the offspring in families where both parents are normal and both parents are affected.

2. Subjects with asymptomatic cholelithiasis will be followed to determine the natural history of the disease.

3. The incidence of gallstones in young females will be determined by follow up of subjects already examined.

Honors and Awards: None

Publications:

Sampliner, R.E., Bennett, P.H., Comess, L.S., Rose, F.A., and Burch, T.A.: Gall Bladder Disease in Pima Indians--Demonstration of high prevalence and early onset by cholescystography. New England J. Med. 283:1358-1364, 1970.

Serial No. NIAMD-EFS-5(c)
1. Epidemiology & Field Studies Br.
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Diet and Nutrition of the Gila River Indians

Previous Serial Number: NIAMD-EFS-6(c)

Principal Investigator: Dr. Thomas A. Burch

Other Investigators: Dr. Peter H. Bennett
Miss Jeanne M. Reid
Dr. G. Donald Whedon
Dr. Max Miller

Cooperating Units: Indian Health Service (PHS)
Case Western Reserve University

Man Years:

Total:	0.3
Professional:	0.1
Other:	0.2

Project Description:

Objectives:

1. To ascertain whether there is an association of dietary characteristics with the presence of diabetes or cholelithiasis in the Gila River Indian Community.

2. To ascertain whether there is an association of dietary characteristics with the incidence of these diseases.

Methods:

1. Samples of the Gila River Indian Community being followed in the various SFSS programs on diabetes, cholelithiasis, and rheumatic diseases were interviewed to ascertain their dietary characteristics.

2. The medical status of the participants will be determined at periodic intervals and possible association of dietary characteristics and development and/or progression of such conditions evaluated.

Major Findings:

Analysis of the dietary data and its relationship to gallbladder disease and diabetes has been completed. No major disease-related differences were found.

Significance of Research:

The lack of difference in the diet of the subjects with diabetes and with gallbladder disease as compared to control subjects makes it unlikely that the dietary factors investigated are of major importance in the causation of either diabetes mellitus or gallbladder disease among the Pima Indians.

Proposed Course:

The study has been completed and is to be published.

Honors and Awards: None

Publications:

Reid, J.M., Fullmer, S.D., Pettigrew, D.K., Burch, T.A., Bennett, P.H., Miller, M., and Whedon, G.D.: Nutrient Intake of Pima Indians: An epidemiologic search for correlation of dietary factors with prevalence of diabetes mellitus and gallbladder disease. Am. J. Clin. Nutrition, In press.

Serial No. NIAMD-EFS-6(c)
1. Epidemiology & Field Studies Br.
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Indivisual Project Report
July 1, 1970 through June 30, 1971

Project Title: Prospective Study of the Complications and Outcome of Diabetic and Prediabetic Pregnancies in the Gila River Indian Community

Previous Serial Number: NIAMD-EFS-1(c) (1967)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Michael J. Shack
Dr. Arthur Dorf
Dr. Max Miller

Cooperating Units: Indian Health Service (PHS)
Case Western Reserve University

Man Years:

Total:	1.6
Professional:	0.2
Other:	1.4

Project Description:

Objectives:

To determine whether pregnancies in prediabetic women are associated with excessive complications during pregnancy or with excessive mortality or morbidity in the offspring.

Methods:

The course and outcome of pregnancies in all female residents of the Gila River Indian Community (Sacaton Service Unit area) will be followed and recorded in a standardized manner.

Infants will be examined to determine the presence of congenital anomalies and other characteristics, and data related to mortality and morbidity will be compiled. The mothers will be given modified glucose tolerance tests 24-48 hours post partum and retested at approximately two yearly intervals to document the development or lack of development of diabetes. At age five years the children will be re-examined to detect any further abnormalities which may have been inapparent at birth.

Major Findings:

Prediabetes and Pregnancy.

COMPARISON OF NORMAL, PREDIABETIC, AND DIABETIC PREGNANCIES

Maternal Diabetes	Number of Pregnancies	Post-Partum 2 Hr GTT Mean (MG/100ML)	Toxemia* Percent	Birth Weight Percent ≥4000G	Congenital Anomalies Percent	Perinatal Deaths Percent
None (Glucose <140)	760	98.3	3.5	8.5	1.1	0.9
Prediabetic	68	105.0	3.1	13.2	2.9	0.0
Diabetic (Glucose >159)	40	198.2	10.5	22.9	5.0	14.3

*Edema + hypertension and albuminuria.

Data from 1070 pregnancies and deliveries collected over the past five years were examined. Fifty mothers with 68 pregnancies were classified as "prediabetic," defined as either both of her parents being diabetic or normal glucose tolerance in the mother with subsequent examinations revealing diminished tolerance. Forty pregnancies were considered "diabetic."

Congenital anomalies were present in 1.1 percent of offspring from normoglycemic mothers but in 5 percent among infants of hyperglycemic mothers. The frequency was not significantly different between the offspring of prediabetic and normal mothers ($p>0.05$). Perinatal infant deaths occurred in 14 percent and 0.9 percent of offspring from diabetic and normal mothers, respectively ($p<0.05$). There were no deaths in infants from the 68 prediabetic pregnancies. The frequency of toxemia was increased in the hyperglycemic mothers. However, the frequency of toxemia in prediabetic and normal women did not differ significantly (30.1 and 32.6 percent respectively). Infant birth weight increased with diminished maternal glucose tolerance and with increasing maternal percent desirable weight.

Neonatal Hyperbilirubinemia. Serum bilirubin levels were determined on unselected Pima and Papago infants born in the Sacaton Hospital during the first four days of life. All infants became jaundiced at some point during this period although none had evidence of hemolytic disease.

BILIRUBIN LEVELS IN PIMA-PAPAGO INDIAN NEONATES

	Serum Bilirubin Level (mg/100ml)		
	No. Exam.	Mean	Std. Deviation
Birth	53	1.3	0.51
Day 1	51	6.2	1.54
Day 2	51	8.5	2.36
Day 3	48	9.3	3.02
Day 4	40	9.2	3.23
Mean Maximum	57	9.9	2.90

Significant increases occurred daily up to the third day. Various factors were evaluated which have been previously implicated in neonatal hyperbilirubinemia, but none correlated with the hyperbilirubinemia. The bilirubin values were approximately twice the levels reported from other studies in the United States. However, they are quite similar to those values reported in Asian infants.

The metabolic derangement which results in physiologic jaundice of the newborn appears to be present in almost all Pima-Papago infants, but its nature is uncertain.

Significance of Research:

Retrospective studies of the characteristics of prediabetic pregnancies have yielded conflicting results. The study of pregnancies among the Pima Indians, who have a high prevalence of diabetes mellitus, provides a unique opportunity to evaluate a larger number of prediabetics prospectively.

Neonatal hyperbilirubinemia represents a common metabolic defect among the Pima Indians which was previously undocumented. The recognition of the condition and its potential hazards are of immediate importance. Because of the extremely high prevalence of neonatal hyperbilirubinemia, its etiology and treatment can be easily studied in this population. The relationship between this abnormality and other metabolic derangements of biliary metabolism found in the Pima Indians, i.e. cholelithiasis, require further evaluation.

Proposed Course:

The pregnancy study will continue in conjunction with the study on the natural history of diabetes mellitus in the Gila River Indian Community. The number of pregnancies recognized to be prediabetic will increase as the mothers studied develop diabetes mellitus.

Honors and Awards: None

Publications: None

Serial No. NIAMD-EFS-7(c)
1. Epidemiology & Field Studies Br.
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Gila River Indian Community Autopsy Study

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Joseph Likos
Dr. Michael J. Shack
Dr. William W. Gough
Dr. Alan L. Metzger
Dr. Arthur Dorf
Dr. Philip M. LeCompte
Dr. Max Miller

Cooperating Units:
Indian Health Service (PHS)
Veterans Administration
Falkner Hospital, Boston, Mass.
Case Western Reserve University

Man Years:

Total:	1.0
Professional:	0.3
Other:	0.7

Project Description:

Objectives:

1. To determine the causes of death, and associated disease processes in members of the Gila River Indian Community being followed in prospective studies on arthritis, diabetes mellitus, cholelithiasis, etc.

2. To ascertain and evaluate the histopathological changes in tissues and cells as they relate to diabetes mellitus. To assess their importance and relationship to clinical findings in members of the Gila River Indian Community upon whom autopsies are performed.

3. Evaluate factors and conditions associated with increased or decreased mortality rate in the Gila River Indians.

Methods:

1. Permission to perform a postmortem examination will be sought from next of kin of all members of the Gila River Indian Community who die in the Sacaton Hospital or Phoenix Medical Center.

2. The autopsy will be performed in a meticulous manner with special emphasis on examination of tissues and organs believed to be affected in diabetes mellitus.

The tissues will be examined using routine and special staining techniques and by light and electron microscopy when necessary.

3. The information will be correlated with the course/progression of clinical abnomalities studied in the same individuals during life.

Major Findings:

Mortality and Causes of Death Among Adult Pima Indians. The mortality experience of Pima Indians of the Gila River Indian Community was examined. The mortality rates for subjects with diabetes was 2.4 times that of subjects without diabetes mellitus.

MORTALITY (EXCLUDING ACCIDENTAL AND VIOLENT DEATHS) AMONG PIMA INDIANS AGED 15 AND OVER (1963-1969)

	MALES	FEMALES
Total Deaths*	112	73
Number with "Diabetes"	57	51
Expected Number of Deaths**	25.2	19.3
Ratio Actual/Expected	2.3:1	2.6:1

*Excludes those in whom diabetic status was unknown.
**Number expected among diabetic population if they had experienced the same mortality rates as the nondiabetics.

When major cause of death was determined it became apparent that the subjects with diabetes had excessive deaths due to renal disease and infection, but had no significant excess of mortality due to coronary heart disease.

KNOWN CAUSES OF DEATH (EXCLUDING ACCIDENTAL DEATHS) IN PIMA INDIANS

PERCENT OF DEATHS DUE TO SPECIFIED CAUSE	NONDIABETIC	DIABETIC
Heart Disease	9	11
Cerebrovascular	12	4
Cancer	33	11
Renal Disease	7	20
Infections	16	24
Diabetic Coma	0	2
Other	23	27

Autopsy Findings:

Evidence of the nature of the renal disease was sought in nineteen consecutive postmortem examinations in adults. Of these nineteen subjects, ten had been shown to have diabetes and nine had been shown not to have the disease. Seven of the ten diabetic subjects (and none of the others) had histologic evidence of intercapillary glomerulosclerosis. All seven had evidence of the diffuse lession, five had exudative changes and four had the pathognomonic nodular changes.

Significance of Research:

This study will result in a more precise clinical-pathologic correlation than has been previously possible in diabetes mellitus. It will enable the prognostic significance of a variety of clinical findings to be assessed. The obvious advantages of an epidemiologic approach will be realized and a more complete understanding of the significance of glucose intolerance and its sequelae will result.

The observation of a high prevalence of histologic evidence of the specific renal changes of diabetes will enable this disorder to be characterized more fully both clinically and histologically.

Proposed Course:

The systematic collection of tissues will continue. As sufficient specimens become available for special study, detailed examinations of various tissues will be made. Further studies of the kidney, using electron microscopy are planned.

Honors and Awards: None

Publications: None

Serial No. NIAMD-EFS-8(c)
1. Epidemiology & Field Studies Branch
2. Metabolic Diseases Epidemiology Unit
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Experimental and Field Studies of Iodine Metabolism

Previous Serial Number: NIAMD-EFS-6(c)

Principal Investigator: Robert L. Vought, M.D., M.P.H.

Other Investigators: Freddie A. Brown, B.S.

Cooperating Units: Laboratory of Nutrition and Endocrinology, Clinical Endocrinology Branch, NIAMD; Environmental Services Branch, Division of Research Services, NIH; Theoretical Statistics and Mathematics Unit, Biometry Branch, NIMH; National Bureau of Standards; and Richmond County Virginia Health Department.

Man Years:

Total:	3
Professional:	2
Other:	1

Project Description:

Objectives: The long-term objectives of the program are: (a) to examine closely the principal environmental factors associated with prevalence of goiter and (b) to study the nature of the metabolic defects responsible for the disease.

Methods Employed: Accomplishment of the objectives has involved bench laboratory research, clinical research and collaborative field studies.

An assay for naturally occurring goitrogens has been satisfactorily developed and extensively used this year. Also, intensive microbiologic studies of a well associated with high goiter prevalence have been done. Routine chemical, microbiologic and pharmacologic techniques continued as before.

Major Findings: In 1964 and 1965 our field studies of endemic goiter indicated an association between goiter prevalence and water pollution. During the year this finding was corroborated by field studies in Greece. These findings spurred a search for goitrogens among metabolic products of

bacteria. Techniques were developed for preparation of cell-free, lyophilized filtrates of broth cultures of organisms isolated from the well referred to above. Many species have been tested and positive results occurred from three of these species: Penicillium sp., Bacillus sp. and Desulfovibrio desulfuricans. Attempts have been made to purify the active material from Penicillium, so far without success. A preliminary report of these findings was presented at the 98th Annual Meeting of the American Public Health Association, October 28, 1970.

In cooperation with the Clinical Endocrinology Branch, it has been established that a red dye (Erythrosine, FD&C Red No. 3) may substantially contribute to the current iodine excess in the U.S. We have been able to show that a portion of the molecule is metabolized as iodide.

The unit is assisting the Nutrition Program, Center for Disease Control at Atlanta, Georgia, in planning and carrying out surveys of goiter prevalence and clinical studies of iodine metabolism.

Publications:

Sibinovic, K.H., Brown, F.A., Pettigrew, K.D., and Vought, R.L.: Lecithin-agar assay for lecithinase antibodies in serum. Appl. Microbiol. 21: 98-103, 1971.

London, W.T., and Vought, R.L.: Infectious goiter. Science 171: 928, 1971.

INFORMATION OFFICE ACTIVITIES

Summary

Fiscal Year 1971

The Information Office conducts the Institute's public information and educational programs, and prepares the bulk of the regular and special program reports required of the Institute by the Congress, and by NIH and DHEW.

Reporting on the Institute's varied and complex activities, which have increased both in scope and depth in recent years, places heavy demands on the information staff. Together with the increased growth of the Institute has been an increase in the number and variety of internal reports required by both the Congress and by the DHEW.

Press Activities:

Several news releases during FY 1971 aroused more than passing interest in the scientific press and led to interviews with the scientists concerned. For example, one release concerned the development by an Artificial Kidney Program contractor of a presterilized, disposable "envelope artificial kidney" for use with the conventional Kiil apparatus. Home dialysis with the new equipment is technically simpler and less time-consuming, and clinical trials of the envelope kidneys have provided excellent results.

Characteristically, a significant amount of Institute press coverage is initiated by members of the fourth estate. A case in point is the University Group Diabetes Program which has aroused much interest and some passion, an NIAMD-supported study which revealed that the anti-diabetes drug, tolbutamide, is no more effective than diet alone or diet and insulin in the treatment of mild, adult-onset diabetes. Seldom a day has gone by since these findings were reported in June, 1970 that the Information Office has not been exposed, directly or indirectly, to the ramifications of this report. Information Office staff members are still responding to inquiries about the UGDP study almost one year after the findings were made public.

Special Events:

Two special events made heavy demands upon the information staff in reporting these activities. The first concerned the celebration of the 20th anniversary of the NIAMD. This entailed, among other things, arranging a science writers' seminar, and a dinner featuring such Federal Government officials as Secretary of Defense Melvin R. Laird, Assistant Secretary of HEW for Health and Scientific Affairs Dr. Roger O. Egeberg, and Dr. William D. McElroy, Director of the National Science Foundation, among others. NIAMD's Dr. Benjamin T. Burton reviewed recent progress in artificial kidney development and the Institute's

Dr. Jesse Roth reviewed recent progress in diabetes research for the science writers. Another highlight of the 20th anniversary celebration was the preparation of a four page insert for the NIH Record, which provided an exhaustive summary of 20 years of NIAMD research in all relevant disease areas. Currently, the Information Office is distributing this insert in response to inquiries about the function of NIAMD.

The second special event concerned the dedication of the 200-bed Phoenix Indian Medical Center in Arizona. The entire fifth floor of the new facility is occupied by a new clinical research section established by the Institute for research on gallbladder disease, diabetes and other disorders prevalent among the Pima Indians of the area. One Information Office staff member covered the dedication in order to assist news media representatives in reporting the event, which received gratifying coverage throughout the State of Arizona.

Publications and Reports:

As a direct result of the UGDP study findings concerning tolbutamide, a fact sheet was prepared to explain in lay terminology the UGDP findings and their implications. In addition, two popular NIAMD publications - "Peptic Ulcer" and "Sickle Cell Anemia" - were revised and updated.

Weekly Reports to the NIH Director, based upon intramural and extramural research accomplishments, are prepared to inform other DHEW professional personnel of noteworthy developments outside of their own particular areas of interest. Typical Weekly Reports during FY 1971 concerned the UGDP study findings, development of the envelope artificial kidney, treatment of rheumatoid arthritis with penicillamine, a possible role for glucagon in diabetes, evidence of the existence of human prolactin, and the laboratory synthesis of both human growth hormone and parathyroid hormone.

More Information Office staff time is allotted annually to preparation of exhaustive special reports and other material in connection with the Congressional appropriation hearings than is allotted to any other single project. During FY 1971 special reports were prepared on arthritis, diabetes, gastroenterology, kidney disease, cystic fibrosis and nutrition, in addition to the Director's opening statement, the budget narrative, and highlights of research. Invariably, a significant amount of time is also spent on collaborating with other Institute information staffers concerning the preparation of a number of reports which involve two or more Institutes.

Public Inquiries:

During Fiscal Year 1971, the Information Office answered 1,843 public inquiries, 295 Congressional inquiries, and 141 press inquiries from news media, either by telephone or by mail, and distributed 144,440 publications. Considerable staff time is allotted to answering inquiries, often at savings to the time of scientific and administrative staff. Questions may be quite specific and may require information not readily available.

With the phasing out of the Diabetes and Arthritis Control Program, Division of Chronic Diseases, the burden of responding to inquiries normally directed to that facility has fallen upon the NIAMD Information Office. This extra work load has necessitated the hiring of several part-time, temporary employees, as well as overtime for several staff members, in order to cope with requests for NIAMD publications and for Diabetes and Arthritis Control Program publications. At the present time the NIAMD Information Office is distributing these publications at the rate of approximately 10,000 per month.

Exhibits:

The Information Office also conducts the NIAMD program of scientific exhibits. This is a prime resource of professional education. NIAMD displayed seven different scientific exhibits at 11 medical and scientific meetings throughout the country, which were viewed by approximately 80,000 physicians, scientists and paramedical people. Institute exhibits on gout, arthritis, artificial kidney, human growth hormone, Lesch-Nyhan syndrome, cystic fibrosis, and other research activities were among those shown. Institute publications and reprints are distributed in conjunction with the exhibits. An updated exhibit on human growth hormone was completed recently, which made its debut at the 1971 meeting of the International Academy of Pathology. Information Office staff members, including the exhibit specialist, all write press releases, reports, etc., as needed. In this manner the NIAMD office can maintain a substantial and varied output with 8 members, including clerical staff.

OFFICE OF THE DIRECTOR

Summary

Fiscal Year 1971

Dr. G. Donald Whedon, Director
Mr. W. G. Baylis, Executive Officer

The Office of the Director has overall responsibility for directing and coordinating basic laboratory research; clinical investigations; extramural activities such as research grants, research fellowships, and training grants, collaborative contract programs; and epidemiologic and clinical field studies. In addition, this Office cooperates with other scientific organizations in coordinating medical research in the Institute's fields of interest; cooperates and maintains liaison with other Institutes and constituents of other Federal agencies; and participates in determining policy governing the National Institutes of Health. It is also responsible for the collection and dissemination of informational material to interested professional and lay individuals and groups.

The continuing review of the Institute's organizational structure to better identify the areas of responsibility has resulted in the establishment of the Artificial Kidney/Chronic Uremia Section and the Scientific Communications Section in the Office of Associate Director for Program Analysis and Scientific Communications. The Phoenix Clinical Research Section was also established in the Metabolic Diseases Branch, Clinical Investigations.

Several appointments and assignment changes have been made in the staffing of some key positions within the Institute this fiscal year: Dr. Lionel M. Bernstein was appointed Associate Director for Extramural Programs; Dr. Scott M. Grundy was appointed Chief, Phoenix Clinical Research Section; Dr. David R. Davies was appointed Acting Chief, Laboratory of Molecular Biology; and Dr. David R. Kominz was appointed Chief of the Section on Bioenergetics, Laboratory of Biophysical Chemistry.

The activities of the Institute's Information Office are included in a separate report which precedes this summary. This year the Information Office has continued its efforts aimed at informing the general lay public of the Institute's mission and activities. Response was made to more than one thousand, eight hundred and forty-three inquiries from the public. Six different scientific exhibits were displayed at eleven medical and scientific meetings throughout the country, and over 144,440 publications were distributed.

Activity in the Program Contract Office continued through F.Y. 1971 at about the same level as the previous year. There was a slight increase in the number of active contracts (from 73 to approximately 80). These contracts were for artificial kidney development, hormone distribution, scientific communications, and augmentation of intramural research.

Coordination continued to be good within the Institute among the various operating elements, and externally with the Research Contracts Branch, Financial Management Branch, the DHEW Patent Advisor, and the contracting community in general.

During the past year eighteen employees have received training in the operation of the remote computer typewriter terminal specifically oriented toward use of the Wylbur text editing computer program and remote computer job entry. In addition to the system developed last year for financial management of contracts, the new Wylbur program is now used for drafting and preparation of manuscripts, collection and maintenance of training records, and personnel data for the Visiting Scientist program. The major accomplishment was the drafting, editing and production of camera-ready copy of the 1972 Congressional Justification of the budget which included both narrative and tabular material requiring nearly half a dozen major modifications.

On November 12, 1970, the Institute celebrated its 20th Anniversary. A science writers' seminar was held, commemorating the event. A banquet was also held at which several notables, including Secretary of Defense Melvin R. Laird, Assistant Secretary of HEW for Health and Scientific Affairs Dr. Roger O. Egeberg, and past and present NIAMD and NIH Directors commended the progress of the Institute during its two decades.

On December 12, 1970, the new Phoenix Indian Medical Center was dedicated in Phoenix, Arizona. The Center was designed to provide NIAMD with the entire 5th floor for clinical research. This facility permits the extension of clinical and laboratory research in disease processes of the gastrointestinal tract, arthritis, diabetes, and various other metabolic diseases, with particular emphasis on those more prevalent among the American Indian population than among the general population of the U.S.

Management emphasis continues to be placed in enhancing training opportunities offered both in-service and through non-governmental facilities. The Personnel Office staff of this Institute completed Part II and Part III of the Basic Ideas in Biology, a training program for nonprofessional laboratory workers. This training program is part of the Institute's overall effort to increase employee productivity through better understanding of how each one's job fits into the work of his section and to fully utilize employees with potential for advancement in career fields related to their interest and abilities. During the year short-term training opportunities were arranged for 103 Secretarial-Clerical employees, 3 supervisory managerial personnel and 95 members of the professional staff.

An NIAMD Committee on Equal Employment Opportunity has been formed. This is a permanent, continuing group organized to provide advice and suggestions to the Director, NIAMD on matters pertaining to Equal Employment Opportunity. This biracial group is made up of employees from each of the major areas within the Institute. A statistical analysis was made of all recruitment and promotion actions over a period of 2 years and it was found that statistically there was no indication of differences in the treatment or handling of whites versus blacks. Several cases have been studied in depth by members of the Committee.

The Committee has been divided into several subcommittees or groups to study Promotions, Training, Recruitment and Counseling and Communication within NIAMD. Several meetings have been held by each subcommittee and oral reports have been made to the full Committee. Written reports will be made and the findings and recommendations of each group will be considered by the total Committee.

Increased emphasis is being placed on publicizing the EEO Program and to assure equal opportunity within the Institute. This item is scheduled for regular discussion at various levels of meetings regularly held within NIAMD. Employee counseling continues to be emphasized with continuing emphasis on training for all levels of employees, during working hours within NIAMD and regular courses offered at NIH and elsewhere outside of working hours. The black employees of the Institute meet at regular intervals. These meetings are attended by black members of the NIAMD-EEO Committee at which time the progress being made is reported and new areas of emphasis are identified. The women of the Institute are also holding regular meetings to discuss the EEO interests of their group.